PRODUCTION AND UTILIZATION OF PHYTOENE, PHYTOFLUENE, AND LYCOPENE TRACERS FOR BIOAVAILABILITY AND BIODISTRIBUTION STUDIES

BY

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DISSERTATION

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Abstract

Tomato product consumption is epidemiologically associated with a decreased risk of cardiovascular disease as well as several cancers, and is most strongly associated with decreased prostate cancer risk. Tomatoes contain a complex milieu of nutrients and bioactive compounds including the carotenoids lycopene (LYC), phytoene (PE), and phytofluene (PF). Much emphasis has been placed on the bioactivity of LYC, but emerging research suggests that the colorless carotenoids, PE and PF, are bioactive and significantly accumulate in human and animal model tissues. To elucidate the absorption and metabolism of these tomato carotenoids, an *in vitro* plant cell culture system for production of isotopically labeled carotenoids was developed, and absorption and biodistribution studies were performed in a relevant animal model, the Mongolian gerbil (*Meriones unguiculatus*).

To enhance ^{14}C-carotenoid production from tomato (*Solanum lycopersicum* cv. VFNT cherry) *in vitro* cell suspension cultures were treated with two bleaching herbicides during the culture incubation, 2-(4-chlorophenyl-thio) triethylamine and norflurazon separately or in combination, to produce varying ratios of PE, PF, and LYC (CPTA, 1:2:18; norflurazon, 11:5:1; combination, 6:1:4, respectively). Treatment with both herbicides resulted in optimal production of all three carotenoids (1.24 mg LYC/L, 1.74 mg PE/L, and 0.31 mg PF/L). Subsequently, cultures were incubated in [^{14}C]-sucrose-containing media to produce labeled LYC, PE, and PF. The impact of the timing of ^{14}C-sucrose addition was evaluated in norflurazon-treated cultures, and adding [^{14}C]-sucrose on day 1 of the 14-d culture incubation cycle to led to a small increase in labeling efficiency compared to adding it on day 7. In short, for optimal label incorporation, cultures should be grown with labeled carbohydrate for the duration of the growth cycle. Further, if primarily PE and PF are needed, norflurazon treatment should be used, for LYC

accumulation CPTA should be administered, and to obtain a mixture of PE, PF, and LYC, cultures should be grown with a combination of CPTA and norflurazon.

A second *in vitro* tomato cell culture study identified high LYC-producing cell line for [^{13}C]-carotenoid production. Different *Solanum lycopersicum* allelic variants for high LYC and varying herbicide treatments were compared for carotenoid accumulation in callus and suspension culture. The *gh* tomato cell cultures produced more PE (3.5 mg/L) without the addition of norflurazon than that previously reported for VFNT cultures treated with norflurazon (2.1 mg/L), making it a preferred culture system for labeled PE production. When the *hp-1*cell cultures were treated with herbicides, they produced greater total carotenoids (3.6-5.2 mg/L) than the previously used VFNT cherry tomato cell line, and therefore *hp-1* cell suspension culture system was chosen for isotopic labeling. When grown with [U]-[^{13}C]-glucose and treated with CPTA for LYC production, *hp-1* suspensions yielded highly enriched ^{13}C-LYC, with 45% LYC in the M+40 form, and 88% in the M+35 to M+40 isotopomer range. In conclusion, the *hp-1* cell line is preferable to other allelic variants or the VFNT cherry cell line for labeled tomato carotenoid production.

Lastly, the gerbil was utilized as a model of tomato carotenoid absorption and bioavailability. The tomato carotenoids PE, PF, LYC, and zeta-carotene (ZC) were all absorbed from a 10% tomato powder diet and were differentially distributed to tissues at physiologically relevant levels compared to those previously reported for human tissues. Further, although LYC was much more concentrated in the tomato powder diet, the tomato carotenoids accumulated in tissues at similar concentrations. When unlabeled PE and LYC doses were provided to tomato powder-fed gerbils (LYC dose, 1.31 ± 0.05 mg in 206 ± 3 μL cottonseed oil; PE dose, 1.00 ± 0.01 mg in 202 ± 3 μL cottonseed oil), PE was more readily absorbed (serum PE increased by

570% versus serum *Z*-LYC 90% and *E*-LYC 40% at 6 hr post-dosing). PE dosing led to greater increases in liver PE at 6, 12, and 24 hours postdosing (81, 68, and 113%, respectively) compared to the respective increase in liver LYC after LYC dosing (-12, 20, and 0%, respectively). In conclusion, although the prominent tomato carotenoids structurally differ only by number of double bonds, absorption and bioaccumulation of these carotenoids differs substantially. These results warrant future studies into the differential bioavailability and metabolism of tomato carotenoids in mammals by utilizing isotopically labeled tracers.

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Thank God every morning when you get up that you have something to do which must be done, whether you like it or not. Being forced to work, and forced to do your best, will breed in you temperance, self-control, diligence, strength of will, content and a hundred other virtues, which the idle never know.

-Charles Kingsley

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CHAPTER 1:
Literature Review

Introduction

Consumption of tomato-based foods is linked to a decreased risk of chronic diseases, especially prostate cancer and cardiovascular disease (CVD). While the red carotenoid lycopene (LYC) appears to be important for disease prevention, other colorless carotenoids, such as phytoene (PE) and phytofluene (PF) found at high concentrations in tomatoes are likely important. However, at this time, little is known about the bioavailability (the proportion of consumed, intact carotenoid that appears in the circulation) bioaccumulation (the amount of consumed, intact carotenoid found in tissues), and metabolism (the chemical modifications to a carotenoid for utilization or clearance) of these red and colorless tomato carotenoids in humans and animal models, largely due to a lack of commercially-available isotopic tracers. Tomato plant cell cultures provide an option for production of labeled carotenoids for use in humans and animals to track their absorption and metabolism.

Tomato consumption prevents chronic disease progression in humans

In 2009, CVD was the primary cause of death for Americans, followed by cancer. Prostate cancer was the most frequently diagnosed cancer in men, and the second leading cause of cancer death after lung cancer (1, 2). Both of these diseases are impacted by nutritional and environmental factors, therefore, nutritional intervention for disease prevention and attenuation is of great interest.

CVD is an umbrella term given for a cluster of diseases, which "includes coronary heart disease (CHD) (myocardial infarction, angina pectoris, coronary insufficiency/death), cerebrovascular diseases (stroke and transient ischemic attacks), peripheral vascular disease,

congestive heart failure, hypertension, and valvular and congenital heart disease (3)." Strong epidemiological evidence has emerged to demonstrate the risk factors for developing CHD include age, male gender, high serum low-density lipoprotein and low high-density lipoprotein cholesterol, cigarette smoking, and diabetes mellitus. In addition to these risk factors, building evidence indicates that lifestyle, genetic factors, and diet are important modulators of CHD risk (3). Over 40% of deaths in Western countries can be attributed to CVD, and this increased mortality compared to non-Western countries may be partially attributable to differences in lifestyle (4).

The pathophysiology of CVD is believed to include such processes as oxidative stress, endothelial dysfunction, inflammation, and vascular remodeling; therefore, reduction of the initial oxidative insults that begin the course of damage is important. Generally, the initial oxidative damage occurs because of excessive reactive oxygen species (ROS). ROS are believed to oxidize LDL, which may eventually lead to atherosclerosis- the precursor condition for stroke and heart attack (3). Dietary antioxidants, such as carotenoids, are hypothesized to act in addition to endogenously-produced antioxidants to diminish the initial oxidative stress which leads to CVD pathology (3). LYC is a powerful antioxidant *in vitro* and some studies examining tomato-based food consumption have shown short-term decreases in LDL oxidation. Additionally, one tomato extract supplementation trial reported a modest hypocholesterolemic effect (3), therefore tomato consumption may reduce CVD risk (5). Several epidemiological studies have suggested that increased LYC concentrations in adipose and serum are inversely related to intimal wall thickness, intima-media thickness, and the risk of myocardial infarction, CVD, and atherosclerosis of the carotid artery and the aorta. A nested case-control study of women found that plasma LYC levels were inversely correlated with CVD incidence over a 7-

year follow-up period, whereas studies of men were more heterogeneous. One nested case-control study demonstrated that plasma LYC levels in men were not associated with CVD risk, while another revealed a positive relationship with myocardial infarction, and a third displayed an inverse association with stroke risk. When dietary LYC was examined in the Women's Health Study, there was a weak inverse association with CVD incidence, however tomato-product consumption was more strongly inversely associated with CVD than dietary LYC (these epidemiological studies are summarized in (5)). In conclusion, a number of studies examining an association between tomato product consumption and risk or markers of cardiovascular disease risk were reviewed, and these indicated an inverse association between CVD and plasma, serum, and adipose levels of LYC (4). Whether LYC is the bioactive component for attenuation of CVD or is just a marker for tomato consumption, however, remains unanswered by these studies.

More robust evidence for a benefit of tomato products and their phytochemicals is likely derived from data regarding cancer. For example, the systematic review by the American Institute for Cancer Research (AICR) and World Cancer Research Fund (WCRF) documents considerable evidence linking tomato carotenoids, specifically LYC, and reduced cancer incidence (6). Indeed, several cohort and case-control studies indicate that foods rich in carotenoids probably protect against mouth, pharynx, larynx, and lung cancers (6). Other evidence suggests that lung cancer (2) is likely reduced with increased dietary and serum or plasma carotenoids (6) and a series of studies in the “smoking ferret” model suggest that LYC may have chemopreventive properties against lung cancer (7). Other evidence and hypotheses exist for LYC and/or tomato phytochemicals in the prevention of skin, liver, and breast cancer (6).

Perhaps the strongest data exists for a relationship between tomatoes, their carotenoids, and prostate cancer risk. Epidemiological evidence from a study of the dietary patterns and disease incidences from the Health Professionals Follow-up Study indicates that tomato sauce, tomato, and pizza intake was significantly correlated with a decreased risk of prostate cancer (8). Clinical intervention and observational trials regarding tomato products and prostate cancer have been reviewed (9, 10) and have shown mixed, but mostly favorable results. Based upon a review of all available human studies, AICR/ WCRF deemed "foods containing LYC probably protect against prostate cancer (6)."

Cancer is believed to be a result of oxidative damage to DNA as well as a result of genetic factors, exposure to environmental toxicants, deregulation of the cell cycle, a loss of cell-cell communication, and altered hormone and growth factor signaling. Various studies have examined the impact of tomatoes and LYC on these parameters with promising results that suggest that tomato products protect DNA, induce phase II detoxification enzymes, modulate hormone and growth factor signaling, induce cell cycle arrest, and increase gap junction communication (reviewed in (11)). In contrast to the measured statement above by the WCRF/AICR specifying foods *containing* LYC, emphasis is often placed on LYC *alone* in research and product marketing. It is important to note that many studies on LYC and health outcomes actually provide human subjects with a mixture of tomato carotenoids but refer to it only as "LYC supplementation." We believe it is of immediate importance to determine the contribution of the other bioactive phytochemical components in tomatoes.

Tomato Nutrients and Bioactives

Tomatoes contain a complex mixture of bioactive components and nutrients making them part of a balanced diet, yet it is difficult to identify a single bioactive compound responsible for

potential health benefits. Tomatoes serve as a dietary source of potassium, folate, and the vitamins A, C, and E (11). Additionally they contain a mixture of carotenoids including LYC, γ-carotene, PE, neurosporene, PF, ζ-carotene, β-carotene, and lutein (12). There are over 600 carotenoids found in nature, and they are generally colorful orange, red, and yellow pigments synthesized by photosynthetic plants, bacteria, and fungi. These tetraterpenes usually consist of 8 isoprene units derived from isopentenyl diphosphate (IPP), the same precursor required for cholesterol synthesis in animals, however in plants carotenoids are synthesized in plastids via the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway rather than the mevalonic acid pathway of cholesterol biosynthesis (13) (Figure 1.1). IPP condenses with dimethylallyl diphosphate (DMAPP) to yield geranylgeranyl diphosphate (GGPP), a C_{20} molecule, which subsequently condenses with another GGPP via phytoene synthase to give the carotenoid precursor PE. Then PE is sequentially, enzymatically desaturated to first yield PF and eventually LYC (13). During early ripening of the tomato fruit, DOXP synthase expression, as well as phytoene synthase mRNA levels increase, while lycopene cyclase (the enzyme responsible for cyclizing LYC to yield β-carotene) levels decrease. Because carotenogenesis is intimately tied to ripening processes, any "biotic or abiotic factor" that impacts tomato ripening "such as genotype, fruit maturity level, cultivation practice, and environmental conditions" will effect the carotenoid profile present in the tomato (14). Carotenoids are not the only compounds impacted by these factors which promote ripening, but other nutrients and bioactives such as tocopherols, folates, phenolics, glycoalkaloids, flavonoids, and vitamin C also vary by ripening stage (14-16)

Prostate cancer and tomato consumption in rats

Based on epidemiological associations for LYC and prostate cancer, our lab sought to determine if LYC alone was responsible for disease prevention or if the mixture of bioactives

found in tomatoes is important. Our lab found tomato powder to be more effective than purified LYC beadlets in both slowing implanted prostate tumor growth or decreasing chemically-induced tumor incidence in rats (17, 18). These findings suggest that LYC alone is not solely responsible for disease prevention and amelioration, but that other components in tomatoes are bioactive.

There are many proposed mechanisms for LYC-mediated cancer prevention such as induction of phase II enzymes, suppression of cell proliferation, antioxidant protection, and upregulation of gap-junction communication (19). To isolate the role of LYC compared to the whole tomato, *ex vivo* assays compared the effect of feeding LYC beadlets to the effect of feeding rats red (LYC-rich) or yellow (LYC devoid) tomato-containing diets on biomarkers of oxidative stress and induction of connexin 43, a gap junction protein (20, 21). In both studies, rats were fed diets for 6 wk supplemented with LYC beadlets (50 mg LYC/kg diet), red tomato powder (50 mg LYC/kg diet), yellow tomato powder (0 mg LYC/kg diet), or the basal diet, which contained 1/3 of the vitamin E requirement. The sera of the rats were added to the media of human prostate cancer cells, and the sera from rats fed yellow or red tomatoes significantly stimulated connexin 43 protein levels compared to the sera from LYC beadlet and control-fed animals (21). Similarly, when heart tissue homogenates from these rats were subjected to lipid peroxidation, yellow and red tomato-fed rats were protected compared to LYC beadlet and control powder feeding (20). Taken together, these studies suggest that a combination of tomato phytochemicals is important in putative anti-cancer mechanisms compared to LYC alone, and further that LYC-free tomato powder is as effective as LYC-containing powder for gap-junction protein stimulation and antioxidant protection.

Phytoene, phytofluene, and lycopene

As discussed above, whole tomato powder was more effective than LYC alone in the previously-discussed animal studies likely because of the many nutrients and phytochemicals found in whole tomatoes, especially two *colorless* carotenoids, PE and PF. For the field of disease-risk reduction with tomato consumption to move forward, we believe PE and PF must be investigated more in depth. These carotenoids have been largely overlooked because they are colorless, however they are present in amounts similar to LYC in fresh tomatoes with concentrations of 2.6 mg LYC, 1.9 mg PE, and 0.8 mg PF per 100 g (11).

Current knowledge of PE and PF in humans is limited to the following observations A.) they are absorbed from tomato foods and extracts and B.) they accumulate in cells and tissues. When subjects consumed ~5 mg PF/d for 4 wk from a tomato juice, they experienced a significant increase of serum and buccal mucosal cell PF which was comparable to LYC accumulation although there was ~ 15-fold more LYC in the juice (22). In a similar study where subjects received the same high LYC tomato juice (75 mg LYC, 5 mg PF, and 6 mg PE/d) for 4 wk, PE and LYC increased in plasma by 0.19 and 0.17 μmol/L while PF increased by 0.45 μmol/L (23). PE and PF have been detected at similar concentrations as LYC, in liver, lung, breast, prostate, colon, and skin tissue as well, notably with PE appearing at levels 4-fold higher than that of LYC in lung tissue (12).

Our lab found that human prostate cancer cells absorb PE and its breakdown products *in vitro* (24). We also found that in rats, PE and PF are absorbed, accumulate more in some tissues than in others, and PF may be absorbed more readily than LYC (25), confirming the interesting results seen in humans provided a low amount of PF daily in high LYC tomato juice (22). When tomato powders of differing PE, PF, and LYC profiles were fed to rats for 7 d, different total

amounts of carotenoids and different ratios of PE, PF, and LYC accumulated in tissues suggesting differential absorption and metabolism of these compounds (26).

With regards to bioactivity, short-term exposure (4-d feeding) to PF, LYC, and tomato-powder feeding decreased androgen status in rats, which could be important for hormone-dependent prostate cancer (27). There is also evidence that PE and PF, like LYC, inhibit LDL oxidation *in vitro*, which may translate to protection from oxidative stress (28). When mouse embryonic fibroblasts (NIH3T3) were transfected with the phytoene synthase encoding gene *crtB* from *Erwinia uredovora*, the phytoene-producing mammalian cells were protected from oxidative stress as well as malignant transformation induced by oncogene transfection (29). Similarly, a transgenic *crtB* mouse showed increased connexin 26 expression, which may translate to increased cell-cell communication important for cancer prevention (30). These results suggest that the bioactivity of PE and PF in humans is probable, but considerably more research is needed to support this assumption.

Tomato carotenoid absorption and metabolism in humans

Carotenoid absorption and metabolism is an actively growing area of research. Several studies have demonstrated that carotenoids are absorbed along with dietary fats; indeed consumption of carotenoids with triglycerides, specifically those with long-chain fatty acids, enhances carotenoid absorption (31-33). Bile is needed for the micellarization and subsequent absorption of carotenoids. Thus, since dietary fat stimulates bile secretion, carotenoid absorption efficiency is enhanced with fat consumption (34). Currently, it is believed that carotenoid absorption into the enterocyte is facilitated *via* the scavenger receptor B type 1 (SRB-1) as well as by cluster determinant 36 (CD36). Within the enterocyte, carotenoids are packaged into

chylomicrons and secreted into the lymph. Carotenoids are shuttled in the plasma by lipoproteins and thus their biodistribution is dependent on proper apolipoprotein function (35).

Pharmacokinetic modeling of LYC absorption (transfer of carotenoids from the gastrointestinal lumen to the enterocyte and then into the blood circulation) and biodistribution from a tomato drink has been informative. One human study indicated that regardless of LYC content in the drink (10mg/79 mL, 30 mg/238 mL, 60 mg/476 mL, 90 mg/769 mL, or 120mg/797mL), the same amount of LYC was absorbed (4.69± 0.55 mg), suggesting saturation of absorptive mechanisms. Different tissue compartments were included in a mathematical model developed to describe LYC absorption and biodistribution. Based on the model generated, the liver was found to be the fast turnover tissue while other tissues that were resistant to LYC depletion, serving as the slow turnover pool (36). Though PE and PF differ from LYC by only a few double bonds, some differences in their absorption and biodistribution have been observed. First of all, when subjects were provided with a high LYC tomato juice for 4 wk (75 mg LYC, 5 mg PF, 6 mg PE in 476 mL juice), they accumulated much higher plasma levels of PF (~0.6 μmol/L) than LYC (~0.2 μmol/L) and PE (~0.2 μmol/L). Additionally, while LYC is primarily found in the LDL-containing blood fraction followed by the HDL fraction, PE and PF are primarily found in the LDL fraction and were relatively higher in the VLDL fraction than was LYC (23). Similarly, when the same high LYC tomato juice was consumed for 4 wk in a different study, LYC and PF attained nearly equivalent levels in buccal mucosal cells despite their disparate levels in the juice (22). The authors suggested that either the absorption efficiency or the metabolism of these tomato carotenoids differ (23). However, it could also be that the absorption mechanisms are saturated for LYC at the 75 mg/d level as evidenced by pharmacokinetic research (36). When a standard, commercially-available (lower LYC) tomato

juice (18 mg LYC, 2.1 mg PE, and 1.1 mg PF/d) was provided for 3 wk to humans, PF and LYC plasma concentrations increased the same amount (138 and 125%) while the increase in PE was lower (110%) (37).

Two carotenoid cleavage enzymes metabolize carotenoids. Carotenoid monooxygenase 1 (CMO1) is well known to centrally cleave pro-vitamin A carotenoids, such as β-carotene, at the 15, 15' double bond to yield vitamin A (38). Carotenoid monooxygenase 2 (CMO2) cleaves β-carotene eccentrically (39), and it is hypothesized to lead to eccentric cleavage of other carotenoids such as LYC (40). In fact, ferret CMO2 expressed in *Spodoptera frugiperda* cells effectively cleaved *all-trans* β-carotene as well as *cis*-LYC isomers (but not *all-trans* LYC) at the 9', 10' double bond (summarized in (19)). Additionally, our lab has detected the LYC metabolites apo-8'-lycopenal and putatively apo-12'-lycopenal in the liver of rats fed LYC for 30 d (41). The CMO2 gene has been detected in an array of human tissues both co-occuring with CMO1 (small intestinal and stomach mucosa, Leydig and Sertoli cells in the testis, kidney tubules, adrenal gland, exocrine pancreas, and epithelium in the eye), as well as being expressed in tissues that do not express CMO1 (cardiac and skeletal muscle, prostate and endometrial connective tissue, and the endocrine pancreas) suggesting that CMO2 has a function independent of VA production (42). Although more information on CMO2 is emerging, very little is known about the carotenoid cleavage products of this enzyme, and it is unknown if PE or PF are cleaved by CMO2.

Pharmacokinetic carotenoid tracer research

Isotopically labeled (^{13}C or ^{14}C) carotenoid tracers allow a single carotenoid dose to be followed within pre-existing, endogenous pools. This is especially important in dietary carotenoid metabolic research since these natural compounds are found widely in the diet of

Americans. It is difficult to completely exclude carotenoids from the diet, as would be desired to conduct conventional pharmacokinetic and metabolic studies. Previously, ^{13}C or ^{14}C tracers of lutein and β-carotene have been utilized to study absorption, plasma appearance, and endogenous pools of these specific carotenoids in humans. Labeled lutein and β-carotene studies have provided information on these carotenoids regarding their absorption kinetics, isomerization of β-carotene during absorption, endogenous plasma pool quantification, and the conversion of β-carotene to vitamin A (43-49). Additionally, deuterium-labeled LYC obtained either by chemical synthesis or from intrinsically labeled tomatoes grown hydroponically were used to determine the relative bioavailability of LYC from steamed and pureed tomatoes versus synthetic LYC in oil. The results showed that synthetic LYC in oil was 3 times more bioavailable (50). Some pharmacokinetic LYC tracer research has been previously performed in rodents. Two studies from our lab focused on the absorption and biodistribution of ^{14}C-LYC in F344 rats (51, 52). When rats were fed 0.25 g LYC/kg diet for 30 d and then gavaged with a single oral dose of ^{14}C-LYC with unlabeled LYC (0.246 mg in oil), 7% of the dose was absorbed and 6% was retained over 168 hr. Radiolabeled LYC was also used to determine that pre-feeding LYC, as opposed to a control diet, which decreased the amount of absorbed LYC (51). To date, no tracer studies on phytoene or phytofluene absorption kinetics, relative bioavailability, biodistribution, or metabolism have been performed in animals or humans and therefore we know very little about these prevalent dietary carotenoids.

Carotenoid metabolism in gerbils

Recently, LYC absorption has been examined in the Mongolian gerbil (*Meriones unguiculatus*). When F344 rats, BALB/c mice, nude mice, and gerbils were provided oral doses of LYC (20 mg/kg BW) every two days for 10 d, gerbils accumulated the greatest hepatic and

plasma LYC. Additionally, in a subsequent experiment, the authors found that gerbils provided with the previously specified dose for 20 d (10 doses) achieved steady state plasma LYC concentration of 597 to 722 nmol/L after 6 days of supplementation (53). Another study sought to compare LYC bioavailability from tomato paste powder, red carrot powder, and purified LYC in oil. Each treatment provided ~60 μg LYC/d to the gerbils for 3 wk. LYC was most bioavailable from the tomato paste powder, followed by the red carrot powder, and lastly the LYC in oil. Further, several thousand times lower levels of LYC were detected in the testes and adrenals than in liver in response to the LYC-containing treatments, showing that carotenoid bioaccumulation patterns in gerbils are more similar to rats than humans. Humans can accumulate up to 9-times the concentration of LYC in those tissues compared to the liver (54).

In addition to LYC absorption, gerbils have proven to be an excellent model for other carotenoids and for vitamin A absorption and metabolism research. Similar to humans, gerbils absorb β-carotene intact, and convert β-carotene to vitamin A at a similar efficiency to humans (55). Gerbils are also useful models for vitamin A status and vitamin A production from carotenoids. The bioefficacy to serve as vitamin A precursors of β- and α-carotene in carrots, cassava, and maize have been assessed using the gerbil as a model (56-60). Additionally, gerbils are good models for evaluating effectors of carotenoid absorption and bioefficacy such as xanthophylls, dietary fat, and fiber (57, 61, 62). Although we understand a great deal about gerbil pro-vitamin A carotenoid uptake and metabolism and are learning more about LYC, currently nothing is known about the absorption and distribution of PE and PF in gerbils.

The gerbil as a model for prostate cancer and hypercholesterolemia

The Mongolian gerbil is not only an excellent model for carotenoid uptake and metabolism but is an established model of lipid metabolism and an emerging model of prostate

cancer. The gerbil can be used to study spontaneous, age-related prostate lesions. Gerbils, unlike other rodents, have a compact prostate (not divided into discrete lobes) similar to humans, but like other rodents they differ in the macro- and microscopic glandular anatomy compared to humans. In aged (18 mo) gerbils, there is an 80% incidence of histopathological alterations mainly in the prostate epithelium. The most frequent alteration was prostate intraepithelial neoplasia (PIN), being found in 47% of the affected prostates, followed by microinvasive carcinomas and adenocarcinomas (63). Similar to previous studies in rats, testosterone in conjunction with chemical carcinogens can be used to induce prostate tumor development in gerbils, therefore, recent research investigated the potential of the gerbil to be used for prostate neoplasia induction and study (64). The carcinogen N-methyl-N-nitrosurea was also used with or without testosterone treatment to induce prostate cancer in gerbils to study prostate cancer progression and the role of extracellular matrix component remodeling in cellular invasiveness (65). The gerbil also was used to examine the effect of androgen depletion on androgen sensitivity in the prostate of senescent animals (66).

The gerbil has the most similar serum lipid profile to humans of any rodent and thus has been used for cholesterol and lipid metabolism research for over 40 years (67). The impact of differing dietary lipid profiles on blood lipids has been elucidated using the gerbil (68-70), with recent emphasis on the effect of *trans* fats and phytosterols on plasma triglycerides and cholesterol (71-74). Given the appropriateness of the gerbil as a model of hypercholesterolemia and prostate cancer, a unique opportunity is presented in using the gerbil as a model for tomato carotenoid consumption and disease prevention.

Biolabeled tracer production from plant cell culture

Further progress on mechanisms of tomato carotenoid metabolism has been hampered because there is no commercial source for isotopically labeled PE, PF, or LYC. Tomato cell suspension cultures have proven to be an effective tool for the production of [^{14}C]-radiolabeled carotenoids, which can be used for pharmacokinetic studies (24). By determining the uptake and metabolism of tomato carotenoids, we can better understand the disease-phytochemical relationship.

Plant cell culture is a useful method for radiolabeled bioactive compound production; grape polyphenols, berry anthocyanins, kudzu isoflavones, red clover isoflavones, and tomato carotenoids have been successfully biolabeled (24, 75-77). Several advantages are offered by using plant cell culture rather than whole plants to accumulate radiolabeled metabolites, such as the ability to target selected tissue sources based on desired secondary metabolite production profiles, shortened culture periods, relative ease of extraction, aseptic growth conditions, and uniform exposure to isotopically labeled carbon sources in the media (76).

In order to enhance *in vitro* carotenogenesis, bleaching herbicides have been utilized (24, 78-81). Bleaching herbicides are chemicals that interrupt the normal biosynthesis of carotenoids, either *in vivo* or *in vitro,* causing a fatal loss of photoprotection in the plant, leading to the destruction of chlorophyll upon light exposure (82). "Bleaching herbicide" is an umbrella term for several classes of herbicides such as substituted trialkyamines, phenylpyridazinone derivatives, and phenyl-substituted 6-methyl pyrimidines (82, 83). Previously, our lab utilized the bleaching herbicide norflurazon to promote [^{14}C]-PE and PF accumulation in tomato cells, and the tracers were used for *in vitro* investigation of their uptake into prostate cancer cells (24). Norflurazon is a phytoene desaturase (PDS) inhibitor, which inhibits the conversion of PE, the

first carotenoid in carotenogenesis, to PF (Figure 1.1) (81). Further, norflurazon causes a concomitant increase in *PDS* expression as well as a modest increase in phytoene synthase *(PSY)* expression, likely due to increased photooxidative stress or a lack of end-product regulation of carotenogenesis (Figure 1.1) (84). Campbell et al. previously determined that cv. VFNT cherry tomato cells produced the greatest amounts of PE (~634 μg/L culture) and PF (~88 μg/L culture) with 0.03 mg norflurazon/40 ml culture in a dimethyl sulfoxide (DMSO) carrier (0.07 % final media concentration), added on day 7 of the 14 day growth period (24).

The bleaching herbicide 2-(4-chlorophenyl-thio) triethylamine (CPTA) has also been widely used in *in vitro* tomato research for the study of LYC accumulation and ripening mechanisms (78-80). CPTA, a synthetic tertiary amine, was first reported to induce LYC accumulation when applied to the surface of several fruits and vegetables that do not normally produce LYC such as oranges, carrots, yellow grapefruit, and sweet potatoes (85). CPTA inhibits LYC cyclase (LCYC) causing LYC accumulation and a lack of downstream cyclic carotenoid production (Figure 1.1). Cyclic carotenoids are believed to feedback inhibit carotenogenesis, therefore a lack of cyclic carotenoids may induce the promoter of the second committed enzyme in the carotenoid biosynthetic pathway, *PDS* (86).

Conclusion

Tomato carotenoids are believed to play a significant preventative role in a variety of diseases, however little is known about their underlying biodistribution and metabolism in tissues. In order to make useful dietary recommendations to Americans for CVD and prostate cancer prevention and management by tomato consumption, human clinical trials must be performed. However, only with substantial knowledge of carotenoid bioavailability, biodistribution, and metabolism in appropriate animal models can such studies be properly

designed. To best observe carotenoid metabolism, sources of isotopically labeled carotenoids must be developed and a suitable animal model for metabolism is needed. For these reasons, methods for producing labeled carotenoid tracers from plant cell cultures were established, and the Mongolian gerbil, an emerging model for tomato carotenoid metabolism, was employed. Research combining the tool of labeled tomato carotenoid tracers and the gerbil model of carotenoid absorption, metabolism, and biodistribution will contribute to this important area of nutritional modifications and disease prevention. The overarching hypothesis laying a foundation for this work is that tomato carotenoids and their endogenously produced carotenoid metabolites are bioactive components that prevent or alleviate chronic disease. Specifically for this dissertation, we hypothesize two prominent, colorless tomato carotenoids, PE and PF, along with red LYC are absorbed from the diet and are biodistributed into a variety of tissues. The major goals of this research are to A.) optimize a tomato cell culture production system for biolabeled carotenoids and B.) characterize tomato carotenoid absorption, bioavailability, and biodistribution in the Mongolian gerbil.

Figures

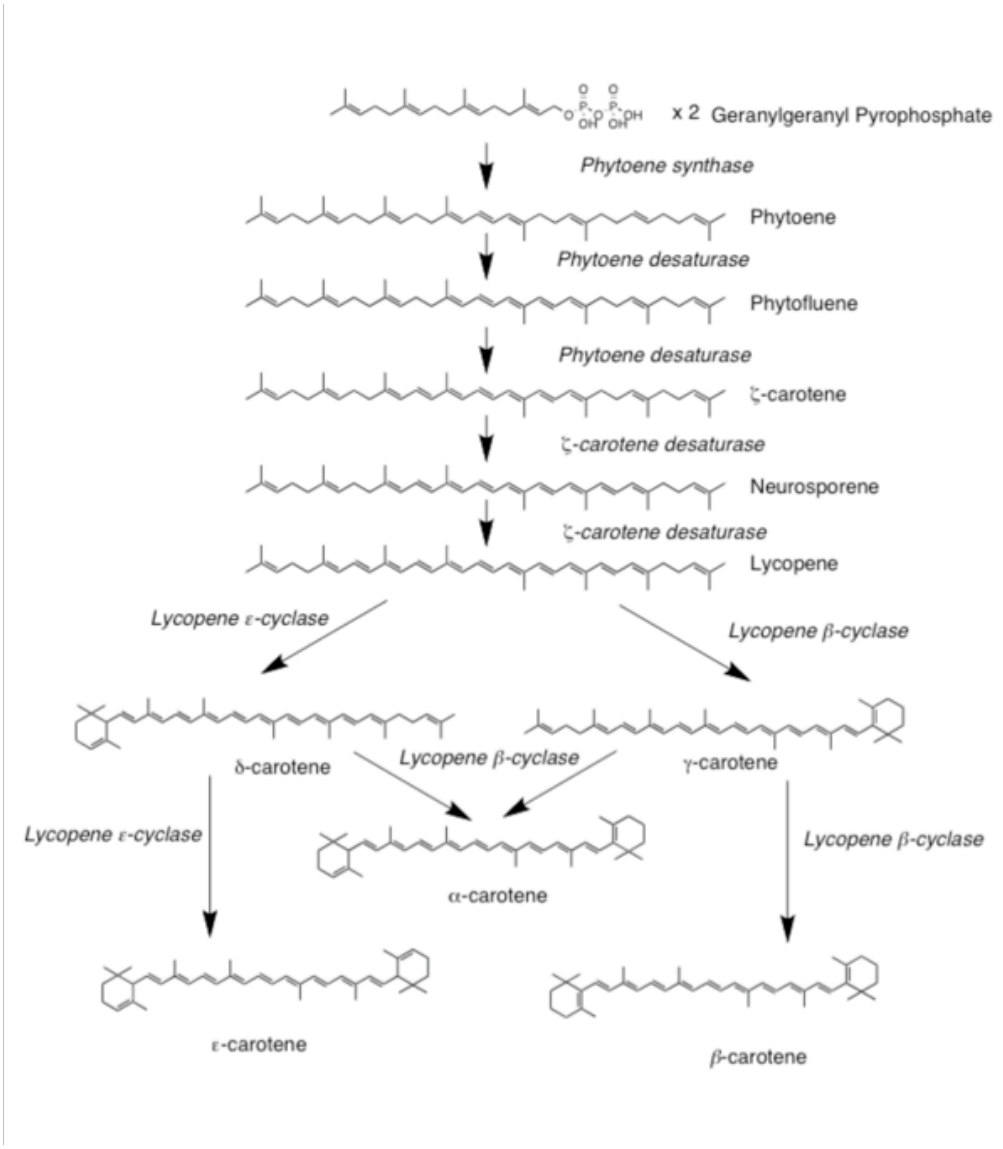

Figure 1.1. Carotenoid biosynthetic pathway found in tomatoes.

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CHAPTER 2:

Herbicide Treatments Alter Carotenoid Profiles for ^{14}C Tracer Production from Tomato (*Solanum lycopersicum* cv. VFNT cherry) Cell Cultures[1,2]

Abstract

Progress in learning about underlying carotenoid bioactivity mechanisms has been limited because of the lack of commercially available radiolabeled lycopene (LYC), phytoene (PE), and phytofluene (PF). Tomato (*Solanum lycopersicum* cv. VFNT cherry) cell cultures have been treated to produce [^{14}C]-PE and PF but with relatively low yields. To increase carotenoid production, two bleaching herbicides were administered during the culture incubation, 2-(4-chlorophenyl-thio)triethylamine and norflurazon separately or in combination to produce varying ratios of PE, PF, and LYC. Treatment with both herbicides resulted in optimal production of all three carotenoids. Subsequently, cultures were incubated in [^{14}C]-sucrose-containing media to produce labeled LYC, PE, and PF. Adding [^{14}C]-sucrose on day 1 of the 14-day culture incubation cycle to norflurazon-treated cultures led to a small increase in labeling efficiency compared to adding it on day 7. Improved culture conditions efficiently provided sufficient ^{14}C-carotenoids for future cell culture and animal metabolic tracking studies.

Introduction

A substantial body of epidemiological evidence suggests that tomato consumption is associated with a decreased risk of a variety of cancers including lung, stomach, and prostate, with the strongest evidence for prostate cancer (1, 2). Tomatoes provide many nutrients to the diet,

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[1] This chapter was the result of a collaboration of the following authors: Nancy J. Engelmann, Randy B. Rogers, Mary Ann Lila, and John W. Erdman, Jr.

[2] Reproduced with permission from Engelmann, N.J.; Rogers, R.B.; Lila, M.A.; Erdman, J.W., Jr. Herbicide treatments alter carotenoid profiles for 14C tracer production from tomato (*Solanum lycopersicum* cv. VFNT cherry) cell cultures. *J. Agric. Food Chem.* 2009, *57,* 4614-4619. Copyright 2009.

including carotenoids, which are 40 carbon, pigmented compounds that range from colorless to red, yellow, and orange (3). The most predominant carotenoid in red tomatoes is lycopene (LYC), followed in concentration by phytoene (PE), phytofluene (PF), β-carotene, α-carotene, and lutein and zeaxanthin (3). These prominent carotenoids are believed to be critical to the efficacy of tomatoes to prevent disease; however the majority of disease prevention research focuses on LYC alone.

To best understand the underlying mechanisms of prostate cancer risk reduction afforded by tomato consumption, a number of investigations into the bioavailability, biodistribution, and metabolism of tomato carotenoids have been undertaken (4-9). Further progress on mechanisms has been hampered because there is no commercial source for isotopically labeled PE, PF, or LYC.

Plant cell culture is a useful method for radiolabeled bioactive compound production, offering several advantages compared to whole plant biolabeling, such as the ability to target selected tissue sources based on desired secondary metabolite profiles, shortened culture periods, relative ease of extraction, and uniform exposure to isotopically labeled carbon sources in the media (10-14).

To enhance *in vitro* carotenogenesis, bleaching herbicides have been used (14-17). Bleaching herbicides interrupt normal carotenogenesis either *in vivo* or *in vitro,* causing a fatal loss of photoprotection in the plant, leading to chlorophyll destruction upon light exposure (18). Previously, the bleaching herbicide norflurazon (NORF) was used to promote ^{14}C-PE and PF accumulation in tomato cv. VFNT cherry cells, and the tracers were used for *in vitro* investigation of their uptake into prostate cancer cells. NORF is a phytoene desaturase (PDS) inhibitor, preventing conversion of PE, the first carotenoid in carotenogenesis, to PF (Figure

2.1) (15). Further, NORF causes a concomitant increase in PDS expression as well as a modest increase in phytoene synthase (PSY) expression, likely because of the increased photo-oxidative stress or a lack of end-product regulation of carotenogenesis (Figure 2.1) (19).

The bleaching herbicide 2-(4-chlorophenyl-thio)triethylamine (CPTA) has also been widely used in *in vitro* tomato research for the study of LYC accumulation and ripening mechanisms (16, 17, 20). CPTA inhibits lycopene cyclase (LCYC), causing LYC accumulation and a lack of downstream cyclic carotenoid production (Figure 2.1). Because CPTA seems to cause an overall increase in carotenogenesis, it was hypothesized that CPTA, in combination with the previously used NORF treatment, would yield greater PE and PF than NORF alone.

Another goal of this study was to enhance the radiolabeling efficiency of targeted carotenoids. Plant secondary metabolites are produced during the growth plateau phase of cell culturing (21). Therefore, examining different [^{14}C]-sucrose-dosing times during the growth cycle may lead to targeted enrichment of carotenoids. The timing effect of adding [^{14}C]-sucrose with NORF on ^{14}C enrichment of PE and PF was examined.

Materials and Methods

Tomato Cell Suspension Cultures and Herbicide Treatments.

Suspension cultures were initiated and maintained according to a previously described method using tomato cv. VFNT cherry calyces for callus initiation (17). Friable callus was transferred from agar-solidified media to solution media and subsequently transferred to fresh media every 2 weeks using published media formulations and culturing methods (14).

To alter the carotenoid biosynthetic pathway, thus optimizing specific carotenoid accumulation, cell cultures were treated with herbicides. A total of 80 mL of fresh media, containing indole-3-acetic acid (5 mg/L) and all *trans* zeatin (2 mg/L) growth regulators in 250

mL Erlenmeyer flasks, was inoculated with 8 mL of spent media and 4 mL of packed cells. A 2 x 2 factorial design for the herbicide treatment experiments was used. On the basis of previous work, herbicide treatments were as follows: cultures were either aseptically treated on day 1 with filter-sterilized aqueous CPTA (74.5 mg/L media, a gift from Betty K. Ishida, USDA-ARS, St. Albany, CA) on day 7 with filter-sterilized NORF (0.75 mg/L media, Syngenta, Greensboro, NC) dissolved in dimethylsufloxide (DMSO), with both herbicides on respective days, or with neither (control treatment) (14, 17). Subsequently, for PE, PF, and LYC radiolabeling, cultures were treated with both CPTA and NORF, and for [^{14}C]-sucrose dose timing experiments, cultures were treated with NORF only.

^{14}C Radiolabeling of Herbicide-Treated Suspension Cultures.

To safely and efficiently ^{14}C-label carotenoids from tomato cell suspension cultures, cells were subcultured into a reduced volume of media (72 mL) and 8 mL of fresh media containing 0.45 mCi/mL uniformly labeled [^{14}C]-sucrose (10 mCi/mmol) (MP Biomedicals, Irvine, CA) was added on either day 1 or day 7 of the 14 day growth period. The final radiolabel-specific activity was 45 μCi/mL fresh media. Cultures were grown in a previously described enclosed Plexiglas radiolabeling chamber equipped with NaOH traps and an air-flushing system to prevent escape of $^{14}CO_2$ from the growth chamber (12). The growth environment was controlled. Rotary shakers were set to 160 rpm. The growing temperature was 22 °C. The cultures were grown in darkness. The chamber air was flushed 4 times per day**.**

In CPTA-treated tomato cv. VFNT cherry cells, the majority of LYC is produced after the end of growth and continues to increase in concentration up to 32 days after inoculation (16). Preliminary studies (data not shown), indicated that when tomato cv. VFNT cherry cultures treated with CPTA were harvested at 14, 21, and 28 days, the LYC concentration increased over

time; however, harvest mass drastically decreased, resulting in an overall lower LYC yield at 21 and 28 days compared to a 14 day culture duration. For this reason a 14 day growth period was selected for further studies. Because NORF is added on day 7 (as opposed to day 1 in the case of CPTA), carotenogenesis in NORF-only treated cultures is very low during the first 7 days of the 14 day growth period. It was hypothesized that adding [^{14}C]-sucrose on day 7 of the growth cycle (rather than initially on day 1) would lead to increased ^{14}C enrichment of PE and PF in NORF-treated cultures.

Three, 14 day long [^{14}C]-radiolabeling runs were conducted. The first examined the carotenoid-labeling efficiency of CPTA- and NORF-treated cells with [^{14}C]-sucrose (12 flasks) provided on day 1. The next two runs (6 flasks/run) compared the effect of [^{14}C]-sucrose dose timing (day 1 versus 7) on PE- and PF-labeling efficiency in NORF-only treated cultures.

Suspension Culture Harvest.

Labeled and nonlabeled cultures were harvested on day 14 using Whatman #4 filters with a Büchner funnel over a vacuum. Filtration was ended, and cultures were collected and weighed when no further liquid was expressed from the funnel for 30 s. Samples of each flask (~1 g) were collected for further carotenoid and ^{14}C scintillation analysis, and the remaining cells were combined for carotenoid preparatory extraction and isolation for future cell culture and animal metabolic studies. When cultures were collected from the radiolabeling chamber, samples were also taken from the NaOH traps and spent media for determination of cell-labeling efficiency.

Carotenoid Extraction of Tomato Cells.

Labeled and nonlabeled carotenoids were extracted from frozen tomato cells as previously described (14) using 0.25-1.0 g cells suspended in 5 mL of ethanol with 0.1% butylated hydroxytoluene and homogenization for 20 s using a tissue homogenizer (Kinematic

PCU1; Brinkmann, Westbury, NY). The cells were heated (60 °C) and vortexed every 10 min for 30 min. Samples were then placed on ice, and then 2 mL of HPLC-grade water and 6 mL of hexane were added. Samples were then vortexed ~30 s and centrifuged for 10 min at 4 °C to separate solvent layers. The carotenoid-containing hexane phase was removed, and the hexane extraction and centrifugation was repeated 2 more times. Reserved hexane was dried under reduced pressure (Speedvac concentrator, model AS160, Savant, Milford, MA) and stored under argon at -80 °C for no longer than 48 h before analysis.

High-Pressure Liquid Chromatography-Photodiode Array (HPLC-PDA) Carotenoid Analysis.

Samples were analyzed using a HPLC-PDA system and methods for carotenoid separation using a C30 reverse-phase column (4.6 × 150 mm, 3 μm, YMC, Wilmington, NC), with mobile phases consisting of methanol, methyl-tert butyl ether, and ammonium acetate (12) for the quantification and isolation of PE and PF from tomato cell suspension cultures (14). All carotenoids were identified and quantified through a comparison to UV spectra, retention times, and standard curves of analytical standards of PE, PF, LYC, BC, LUT (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany), ζ–carotene (ZC), and δ-carotene (DC) (Carotenature, Lupsingen, Switzerland) with published extinction coefficients (22). Radiolabeled PE, PF, and LYC were first separated using a method developed by Craft (23) on an HPLC-UV/vis Varian Prostar dual wavelength detector with Dynamax, SD-200 pumps (Varian, Palo Alto, CA), quantified, and collected. Roughly equivalent amounts of carotenoids (generally between 100 and 200 ng) were then repurified using the method described earlier using a C30 column and the HPLC-PDA system. Carotenoid fractions were collected according to characteristic retention times and quantified on the basis of authentic standards, and solvents were removed under argon.

Biosafe II liquid scintillation cocktail (20 mL) (Research Products International Corp., Mount Prospect, IL) was first added to the dried carotenoid fractions, held in darkness overnight, and analyzed for ^{14}C content using a Beckman liquid scintillation counter, model LS-6500 (Bakersfield, CA).

Analysis of ^{14}C Accumulation in Samples and System-Labeling Efficiency.

Known amounts of labeled carotenoids, media, or NaOH traps were added to BioSafe II (20 mL). Tomato cells (0.05 g) were solubilized in glass scintillation vials using 1 mL of TS-2 tissue solubilizer (Research Products International Corp., Mount Prospect, IL) for 6 h at 50 ˚C with vigorous mixing by vortex every 30 min to ensure that all cells were dissolved. This mixture was bleached with 1 mL of 30% H_2O_2 and neutralized with 0.5 mL of glacial acetic acid. Samples were held at room temperature for 45 min, and then scintillation cocktail was added. The samples were then kept in darkness overnight. ^{14}C content was analyzed by scintillation counting the following day.

Statistical Analysis.

Treatment group differences were determined using analysis of variance (ANOVA) procedures when the assumptions of ANOVA were met, otherwise the Wilcoxon and Kruskal-Wallis nonparametric tests were used to evaluate group differences using the statistical analysis software SAS v. 7.1 (SAS Institute, Inc., Cary, NC).

Results

Herbicide-Induced Carotenoid Accumulation.

Herbicide treatments did not affect cell biomass, where the average yield for all cultures was 153 ± 11 (standard error of the mean, SEM) g of tomato cells/L (n = 12). Carotenoid yields, however, were enhanced by herbicide treatments. CPTA alone or in combination with NORF

resulted in the greatest total carotenoid (combined LYC, PE, and PF) yields (2.86 and 3.28 mg/L, respectively) followed by the NORF treatment alone (2.29 mg/L). Control cultures had substantially lower total carotenoid yield (0.017 mg/L) (Figure 2.2). When herbicide treatments were compared, the combination treatment produced significantly more LYC than the NORF treatment (1.24 versus 0.04 mg/L; p= 0.05) and more PE than the CPTA treatment (1.74 vs. 0.14 mg/L; p=0.05). CPTA treatment led to significantly greater LYC accumulation (2.49 mg/L) than NORF or control treatments (0.04 and 0.04 mg/L; p<0.001 and p=0.02). NORF caused greater PE and PF accumulation (2.06 and 0.18 mg/L, respectively) than control (0 and 0.01 mg/L, respectively; p=0.02 and 0.02) and more PE than CPTA treatment (0.14 mg/L; p=0.034) (Figure 2.2). The effects of herbicide treatments on other minor, quantified carotenoids (LUT, BC, DC, and ZC) are presented in Table 2.1.

[^{14}C]-Carotenoid Yields from Tomato Cell Cultures Treated with CPTA and NORF.

Cultures treated with CPTA (day 1) and NORF (day 7) and dosed with [^{14}C]-sucrose (day 1) were harvested and weighed after vacuum filtration, and the average mass yield was 151 ± 2 (SEM) g/L (n = 12). Upon harvest, samples of culture media, NaOH traps, and system cleanup rinse water analyzed by scintillation counting indicated that 52, 17, and 4% of the initial [^{14}C]-sucrose dose were retained in these system compartments. A total of 18% of the initial ^{14}C dose accumulated in tomato cells and an estimated 0.02% were deposited in combined PE, PF, and LYC. The remaining 9% of the initial [^{14}C]-sucrose was likely distributed throughout all experimental compartments (Figure 2.3). The specific activity of the carotenoids analyzed ranged from 0.11 to0.22 μCi/umol (Figure 2.4A). Phytoene was the most abundant source of radiolabeled carotenoid (2.6 nCi/g cells), followed by all-(*E*)- and 5-(*Z*)-LYC, other LYC (*Z*)-isomers, and PF (2.3, 0.8, and 0.4 nCi/g cells, respectively) (Figure 2.4B).

Labeling Efficiency of Cultures Dosed with [^{14}C] Sucrose on Day 1 or Day 7.

To examine the effect of [^{14}C]-sucrose dose administration timing on carotenoid enrichment, labeled sucrose was added to NORF-treated cell cultures on either day 1 or 7 of the 14 day growth cycle. In this trial, cultures dosed on day 1 yielded 167 ± 3 (SEM) g/L (n=6) fresh mass and cultures dosed on day 7 averaged 174 ± 2 (SEM) g/L (n = 4). Dosing on day 1 vs. day 7 led to a small increase of ^{14}C content in both PF and PE, causing a 69% and 38% increase in specific activities, respectively. Labeled carbon distribution throughout experimental compartments was similar between the two radiolabeling runs (Figure 2.5). Therefore, despite the slightly higher biomass with day 7 dosing, the higher ^{14}C content acquired with day 1 dosing justifies selection of day 1 dosing for subsequent work.

Discussion

Experiments investigating the effect of CPTA and/or NORF on carotenoid content of tomato cell cultures revealed several key points: (a) all herbicide treatments increased total carotenoid content; (b) the combination treatment unexpectedly produced LYC even in the presence of NORF; (c) unexpected carotenoids were detected; and (d) unique mixtures of carotenoids were efficiently produced with each herbicide treatment. The use of successive CPTA (day 1) and NORF (day 7) treatments in a continuous growth cycle allows for the most robust production of a useful mixture of carotenoids for subsequent radiolabeling studies. In contrast, single herbicide treatments would be more effective in maximizing the yield of specific [^{14}C]-labeled carotenoids.

Bleaching herbicides increase total carotenoid content in tomato, pepper, and daffodil plants as well as tomato cell cultures (14, 16, 17, 19, 20, 24-26). The results from the current study confirmed either or both herbicides led to a greater total carotenoid yield than untreated cultures (135-193-fold). For NORF treatment, this has been attributed to either an upregulation of the

metabolic pathway to compensate for a lack of photoprotective carotenoids or increased photostability of carotenoid precursors compared to their downstream products with longer polyene chains (24). The findings from the current report support the first hypothesis but not the second. In the current study, cell culture experiments were carried out in darkness, where photostability would not be a contributing factor in carotenoid accumulation. With regard to increased carotenoid accumulation in tomato cultures with CPTA treatment, this may occur because of an upregulation of carotenogenesis in response to decreased BC or its derivatives, which may normally feedback inhibit upstream carotenogenic enzymes (27). This hypothesis has been supported by increased PSY, PDS, and LCYC mRNA expression and protein levels observed in carotenogenic daffodil flowers treated with CPTA (25).

Second, it was hypothesized that LYC biosynthesis would be blocked in cultures treated with CPTA and NORF in combination, causing a substantial elevation of PE and PF accumulation compared to NORF alone. The results did not support this hypothesis, showing instead that the combined and CPTA treatments alone resulted in similar total major carotenoid content. Surprisingly, the combination-treated cells accumulated 37% of the amount of LYC as that found with the CPTA alone treatment. Tomato cell cultures treated with CPTA appear to synthesize LYC steadily throughout the growth period (17) thus, the observed LYC was most likely produced in the first 7 days of the 14 growth period, while further LYC synthesis was most likely halted with NORF treatment on day 7.

In addition to detecting PE, PF, and LYC, we detected BC, DC, and LUT in small amounts (Table 2.1). This was surprising because CPTA is a LCYC inhibitor; however, the level of CPTA provided may have been less than that necessary for complete LCYC inhibition, or there may be an alternate pathway for carotenoid cyclization. ζ-carotene accumulation increased

in NORF-treated cells compared to the control, which is also unexpected because NORF is a PDS inhibitor (Figure 2.1). Because PF was present at approximately 1/10 the amount of PE, and ZC was present at roughly 1/10 of PF, the chosen NORF dose caused inhibition of PDS, although it was incomplete.

Lastly, different herbicide treatments led to different mixtures of carotenoids. The combined herbicide treatment (NORF + CPTA) led to excellent production of all three tomato carotenoids of interest (Figure 2.2). This novel method of producing carotenoids was thus chosen for subsequent radiolabeling studies. Tailored mixtures, predominant in one or another of the target carotenoids, could be encouraged by further alterations of the herbicide treatment.

The current studies led to several improvements to the previously established carotenoid radiolabeling system (14). Previously, harvest yield obtained from tomato cv. VFNT cherry cell cultures was ~ 70 g/L, while the average for the current experiments was nearly double (~ 165 g/L). This difference was likely due to adaptation of the cultures over time, in that the tomato suspension cultures had been continuously cultured over a period of 12-24 months longer than the time of previous experimentation. Productivity differences between experiments could be responsible for the higher carotenoid yields and lower enrichments of the carotenoids, such that more ^{14}C was channeled into other biosynthetic pathways than carotenogenesis. Between the two current experiments, different ^{14}C distribution in the experimental compartments was also observed, which is likely attributed to later runs accumulating more cell mass (see Figures 3 and 5). Contrary to the hypothesis, adding [^{14}C]-sucrose later in the growth period did not significantly enhance the specific activity of PE and PF but instead led to lower ^{14}C enrichment. It is concluded that tomato cells should be grown with [^{14}C]-sucrose for the full growth period

and that the combined CPTA and NORF herbicide treatment induces the production of all three major tomato carotenoids.

The methods described here for increasing total carotenoid yield, improving carotenoid profiles, and increasing carotenoid specific activity, along with a recently improved tomato cell extraction protocol (28), enhance the efficient production of radiolabeled carotenoids. In addition, these results demonstrate that changing herbicide treatments can alter carotenoid profiles. For example, lowering the NORF dose could potentially reduce efficiency of PDS inhibition and allow for a greater accumulation of PF. One future direction of interest would be to use a high LYC-producing tomato cell line to further increase overall carotenoid-producing potential. The methods described here could also be used to produce stable isotope-labeled tracers for human metabolic research, using [^{13}C]-glucose as the primary carbon source for the cultures.

Safety

All radiolabeled materials were handled according to the University of Illinois Division of Research Safety Radiation Safety Manual.

Acknowledgement

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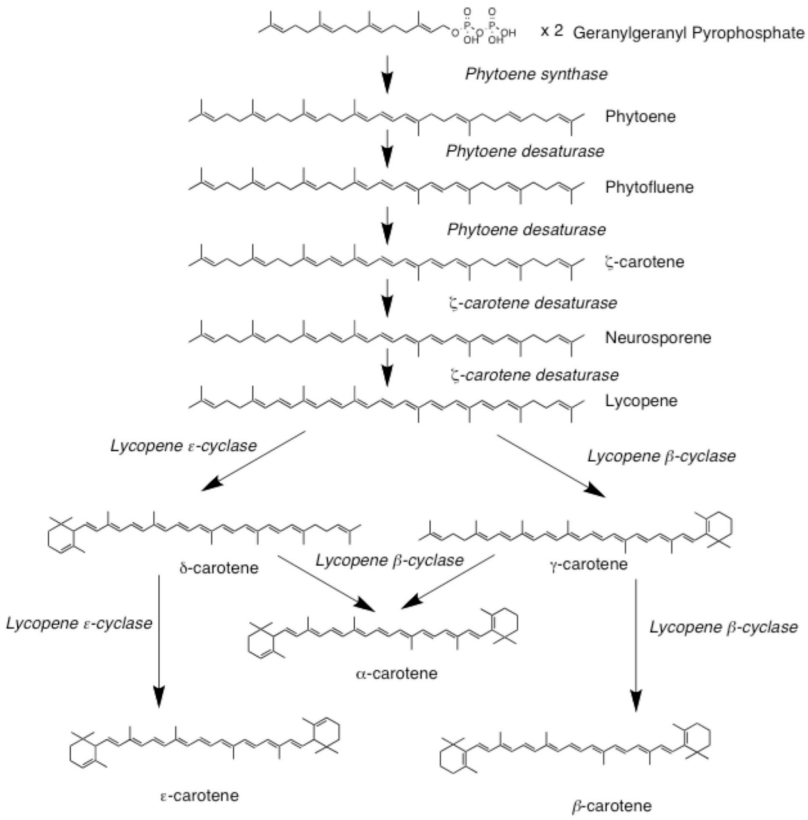


Figure 2.1. Tomato carotenoid biosynthetic pathway.

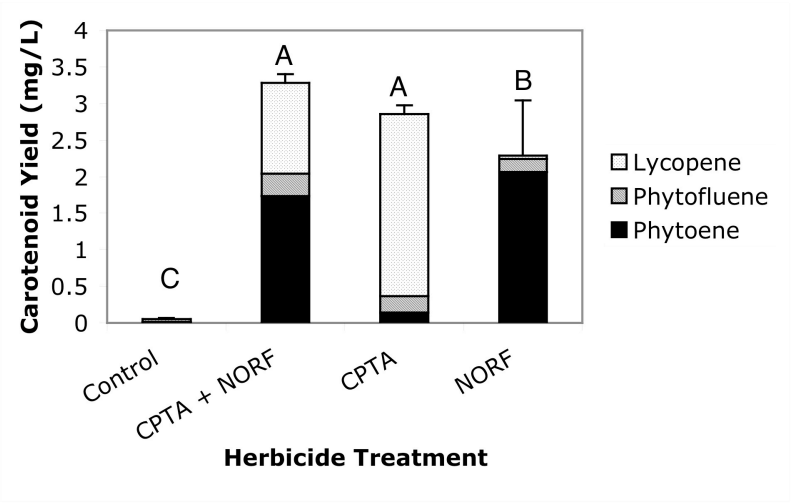


Figure 2.2. Effects of herbicide treatments on production of three carotenoids from tomato cv. VFNT cherry cell culture grown for 14-d. See the Materials and Method section for details of treatments. Bars with different letters are significantly different according to Kruskal-Wallis test (α= 0.05). Error bars represent SEM for combined PE, PF, and LYC (n = 3 trials, analyzed 3 flasks/trial in duplicate).

Figure 2.3.

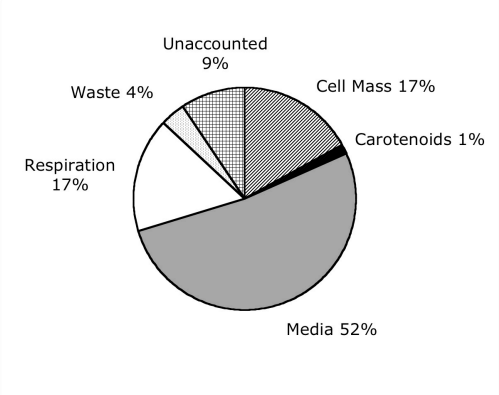
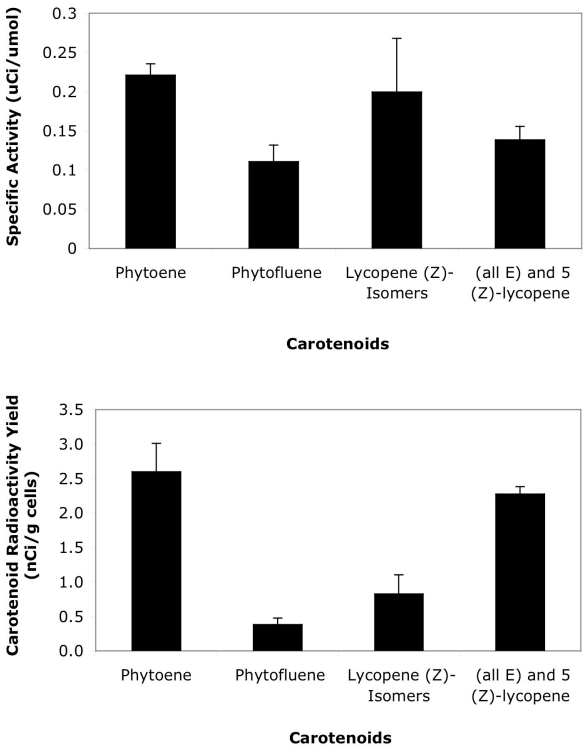


Figure 2.3. Percent recovery of ^{14}C provided on day 1 of a 14-d growth period to tomato cell cultures treated with CPTA on day 1 and NORF on day 7.

A.



B.

Figure 2.4. (A) Carotenoid specific activity and (B) radioactivity yield of carotenoids from CPTA- and NORF-treated cell cultures. [^{14}C]-Sucrose was provided on day 1 of a 14 day growth period. Error bars represent standard error of the mean. n = 3 culture flasks analyzed in duplicate for each carotenoid.

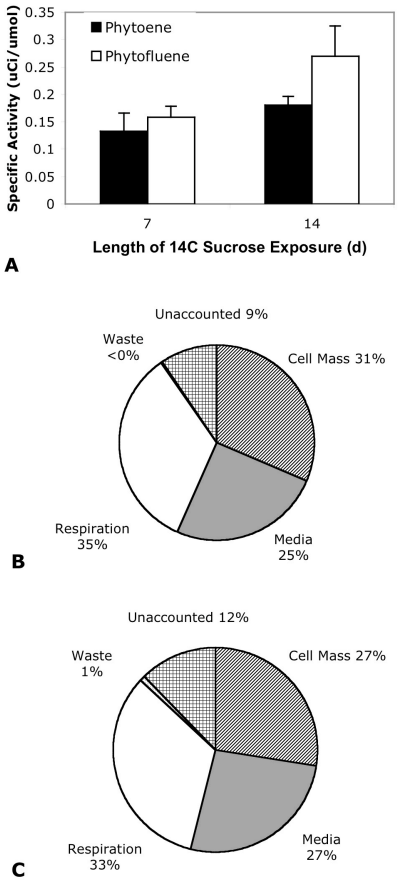


Figure 2.5. Carotenoid specific activity (A) and ^{14}C distribution in compartments of cultures treated with [^{14}C]-sucrose on day 1 (full growth period) (B) or day 7 of the growth period (day 7-14) (C).

Table 2.1. Yield of minor carotenoids following herbicide treatment of tomato cv. VFNT cherry cell cultures after a 14-d growth period. Data are averages of 2 trials, each analyzing 3 flasks, in duplicate.

	(ug/L)			
Treatment	***β-carotene***	***ζ-carotene***	***Lutein***	***δ-carotene***
Control	16.3	0.709	16.6	0.00
CPTA	9.17	1.12	7.53	0.00
NORF	7.64	20.7	13.5	10.2
NORF and CPTA	7.17	26.8	10.1	1.64

CHAPTER 3:
Screening and Selection of High Carotenoid Producing in Vitro Tomato Cell Culture Lines for [^{13}C]-Carotenoid Production[34]

Abstract

Isotopically labeled tomato carotenoids, phytoene, phytofluene, and lycopene, are needed for mammalian bioavailability and metabolism research but are currently commercially unavailable. The goals of this work were to establish and screen multiple in vitro tomato cell lines for carotenoid production, test the best producers with or without the bleaching herbicides, norflurazon and 2-(4-chlorophenyl-thio)triethylamine (CPTA), and to use the greatest carotenoid accumulator for in vitro ^{13}C-labeling. Different *Solanum lycopersicum* allelic variants for high lycopene and varying herbicide treatments were compared for carotenoid accumulation in callus and suspension culture, and the *hp-1* cell suspension culture system was chosen for isotopic labeling. When grown with [U]-^{13}C-glucose and treated with CPTA, *hp-1* suspensions yielded highly enriched ^{13}C-lycopene with 45% lycopene in the M+40 form and 88% in the M+35 to M+40 isotopomer range. To the authors' knowledge this is the first report of highly enriched ^{13}C-carotenoid production from in vitro plant cell culture.

Introduction

Frequent tomato consumption is believed to contribute to a balanced diet as well as reduce chronic disease risk, most notably prostate cancer and cardiovascular disease (1). Tomatoes are a rich source of vitamins and minerals, as well as pro-vitamin A and nonpro-

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[3] This chapter was the result of a collaboration of the following authors: Nancy J. Engelmann, Jessica K. Campbell, Randy B. Rogers, S. Indumathie Rupassara, Peter J. Garlick, Mary Ann Lila, and John W. Erdman, Jr.

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vitamin A carotenoids. Carotenoids are lipophilic tetraterpenes with conjugated double bonds, which are responsible for the bright orange, yellow, and red colors seen in nature (2). Raw, red tomatoes contain substantial amounts of the red carotenoid lycopene (LYC), the colorless carotenoids phytoene (PE) and phytofluene (PF) (9270, 1860, and 820 μg/100 g, respectively), and smaller amounts of cyclic carotenoids and xanthophylls such as β-carotene and lutein (230 and 80 μg/100 g, respectively) (3), although these concentrations can vary substantially by ripeness and cultivar.

The health protective effects of tomatoes are widely believed to be due to carotenoid bioactivity; however, the exact mechanisms responsible are debatable (1, 4). An array of LYC metabolic and oxidative products have been detected in both tomato-based foods and human serum, but it remains unclear whether these products are endogenously produced or absorbed from the food products (5). PE and PF are absorbed from foods and are present in human plasma after 4-weeks of tomato juice consumption (6) and also show different bioaccumulation patterns than LYC in rats in response to a 4 wk tomato powder feeding regimen (7). The overarching questions regarding carotenoid bioactivity are (A) are intact carotenoids or metabolic and/or oxidative products of these carotenoids bioactive? (B) Is LYC solely responsible for bioactivity, or are the other colorless carotenoids, PE and PF, also bioactive? In order to answer these questions, biolabeled carotenoids are necessary for animal and human feeding studies to trace the differential absorption and metabolism of these prominent tomato carotenoids.

Plant cell culture is an efficient tool for producing labeled plant secondary metabolites. In vitro plant methodologies allow for rigorously controlled growing conditions, reliable biochemical responses, short-growth periods, targeted production of desired phytochemicals, simplified extraction, and directed utilization of costly isotopically labeled carbohydrates (8). In

particular, this approach is well suited for labeling secondary metabolites that are found in only plant fruit tissues such as tomatoes and berries, where whole plant labeling *via* enriched water or carbon dioxide would be wasteful and time consuming. Whole plant, or intrinsic, labeling, on the other hand, is well suited for plants that can be grown quickly, and the *entire* plant can be used as a source of the desired phytochemical (for an example of intrinsic labeling, see ref 9). Previously, tomato carotenoids, red clover and kudzu isoflavones, and grape and berry polyphenols have been successfully radiolabeled (i.e. ^{14}C) using in vitro plant cell cultures for utilization in animal phytochemical metabolism research (8, 10-14), and grape flavonoids have been ^{13}C-labeled (15, 16). However, this technology, to the authors' knowledge, has not yet been applied to the production of ^{13}C-labeled carotenoids for mammalian metabolic research.

Two general approaches exist for the efficient production of secondary metabolites from plant cell cultures. First, plants known to hyper-produce a desired phytochemical can be used to derive an in vitro cell line, and that cell line can be evaluated for its phytochemical production profile. Second, elicitors or enzyme modulators can be used to induce or enhance secondary metabolite production and accumulation in in vitro cultures (17). LYC, PE, and PF accumulation in tomato cell culture can be enhanced by treating with the bleaching herbicides norflurazon and/or 2-(4-chlorophenyl-thio)triethylamine (CPTA) (10, 11). Norflurazon inhibits phytoene desaturase, leading to an accumulation of PE and PF, while CPTA inhibits lycopene cyclase, leading to an increase in LYC accumulation (10).

The goal of the following work was to establish tomato cell lines for the efficient production of ^{13}C-carotenoids found in tomato fruit for nutritional research. A screening program was implemented to identify high LYC and high PE and PF accumulating cell lines then their carotenoid production in response to herbicide treatments tested (Figures 1A and B). The

ghost (*gh*) phenotype tomato was used to derive plant cell cultures to investigate their potential to produce PE and PF (Figure 3.1A). The *gh* plant is deficient in plastid terminal oxidase (PTOX), which is a plastoquinone-O_2 oxidoreductase that likely serves as a necessary cofactor for carotenoid dehydrogenases. The lack of PTOX, therefore, interrupts efficient carotenogenesis (18). There are nine genetic loci known within tomatoes that control fruit pigmentation; therefore, several putative high LYC producing tomatoes were selected to be scanned for in vitro LYC production. *Solanum lycopersicum* plants carrying *hp-1, hp-2^dg, B^og* and *B^og^c* alleles in these loci previously reported to have enhanced LYC accumulation were scanned and compared to the previously established VFNT cherry tomato cell line, a line used for the production of radiolabeled carotenoids and in vitro carotenogenesis studies (10, 11, 19). An additional wild species of tomato, *Solanum pimpinellifolium*, was also selected for its elevated LYC accumulation (19). The best performing high LYC cell line was then grown with [U]-^{13}C-glucose to produce ^{13}C-LYC, which was purified and analyzed for isotopic enrichment (Figure 3.1B).

Materials and Methods

Ghost Plant Material and Culture Initiation

Seeds from *gh*/+ tomato (*Solanum lycopersicum* cv. Mill, donated by Wendy White) were germinated under very low light (9 μE $m^{-1}s^{-1}$), and after 5 days, low irradiance was maintained (~80 μE $m^{-1}s^{-1}$) to allow for viable variegated *gh* (*gh*/*gh)* seedlings since higher light intensities cause a lethal white-leafed phenotype (20). The seedling genotype was identified by the presence of either purple shoots and variegated or white leaves (*gh*/*gh; gh*), or green leaves (+/+ or *gh*/+*;* wild-type) (20). Stem and leaf segments were collected at 4 weeks, and flower buds at 11 weeks from greenhouse-grown plants for disinfestation by dipping in 70% aqueous ethanol,

immersing in 7.5% sodium hypochlorite with one drop of Tween-20 (Sigma-Aldrich, St. Louis, MO) for 15 min, and rinsing with sterile distilled water for 5 min. Explants were placed onto 40 mL of sterile, agar-solidified Murashige and Skoog salt-based media containing the growth regulators 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine (maintenance media) for tomato callus induction as previously described (11) in GA-7 cubes (Magenta Corporation, Chicago, Illinois). During callus induction, *gh* stem and leaf-derived calluses were transferred to fresh solidified media after 35 days, then subsequently every 21 days. To promote healthy callus growth, *gh* flower bud-derived callus was transferred to fresh solidified media after 23 days, 12 days later, 9 days later, and subsequently every 21 days. Leaf segments and flower buds from wild-type plants were transferred to fresh solidified media after 41 days and subsequently every 21 days. Cell suspension cultures were initiated with 2 g of friable and the callus added to the same callus induction media as mentioned above, formulated without agar. Cell suspension cultures were subcultured every 2 weeks by the transfer of 6 mL aliquots of established cultures (2 mL of packed cells with 4 mL ofspent media) to fresh maintenance media.

Carotenoid Evaluation of Different Ghost Genotypes in Callus and Cell Suspension Cultures.

For carotenoid analysis, proliferating *gh* and wild-type callus cultures were initiated with 2 g callus/40 mL of solid maintenance media in GA-7 cubes (n = 4-6) and were harvested after 3 weeks. Harvested samples were stored at -80 ˚C until carotenoid extraction and HPLC analysis. Carotenoid yield was evaluated for *gh* cell suspension cultures grown in either liquid maintenance media or the previously described carotenoid production media containing the growth regulators indole-3-acetic acid (5 mg/L) and *all-trans*-zeatin (2 mg/L) (11). Suspension cultures were continuously maintained at 2 week subculture intervals by inoculating either 40

mL of maintenance media in 125 mL flasks with 6 mL of established cultures or 80 mL of carotenoid production medium in 250 mL flasks inoculated with 12 mL of established cultures (4 mL of packed cells and 8 mL of spent media) and were grown (n = 5/media formulation) for one 2 week growth period and then harvested.

High LYC Cell Line Plant Material and Culture Initiation

Seeds of *Solanum pimpinellifolium* (LA0376) and *Solanum lycopersicum* cultivars ['Ailsa Craig' *hp-1* (LA3538), 'Manapal' *hp-2^dg* (LA2451), 'Ailsa Craig' *B^og* (LA3311), wild-type 'Ailsa Craig' (LA2838A), and wild-type 'Manapal' (LA3007)] were obtained from the Tomato Genetics Research Center, UC-Davis, CA, USA. *Solanum lycopersicum* 'Suncoast' $B^{\wedge}og^{c}$ seeds were obtained as a gift from Jay Scott and 'VFNT cherry' seeds from Betty Ishida. Germinated seeds were allowed to reach maturity in a greenhouse. Flower buds and leaves were used to generate callus cultures as described above. Callus was subcultured every 4 weeks for at least 2 growth cycles until friable calluses developed. Transfer of 2 g of callus to 40 mL of maintenance media initiated liquid suspension cultures. Cell suspensions were subcultured every 2 weeks as described above.

Herbicide Treatment Experiments

Cultures were either treated aseptically on day 1 with filter-sterilized aqueous CPTA (donated by Betty K. Ishida, USDA ARS, St. Albany, CA) (0.0745 g/L media) (10) and/or on day 7 with filter-sterilized norflurazon (Syngenta, Greensboro, NC) dissolved in dimethyl sulfoxide (0.06 mg/80 mL media) as previously published (11). To investigate the potential for increasing carotenoid accumulation in *gh* and wild-type cell suspension cultures, either norflurazon or CPTA was added to *gh* and wild-type cultures. Herbicides were provided to *gh* and wild-type cell suspension cultures in 80 mL of the carotenoid production medium. High

LYC tomato cell lines were also treated with CPTA to stimulate LYC accumulation either by aseptic addition to freshly autoclaved callus carotenoid production media before solidification or to cell suspension cultures in liquid carotenoid production media. Callus cultures were grown with or without CPTA in the solid media for one 4 week growth cycle and suspensions for 2 weeks. The *hp-1* tomato cell line was treated with CPTA and/or norflurazon, as specified, to investigate the potential to manipulate the carotenoid accumulation profile.

Callus and Cell Suspension Culture Harvest

Callus cultures were harvested by careful separation of the callus from solid media using metal spatulas and weighed, and fresh mass was recorded. Labeled and nonlabeled cell suspension cultures were harvested using Whatman #4 filters with a Büchner funnel over a vacuum. Filtration was ended, and cells were collected and weighed when no liquid was expressed from the funnel for 30 s. All collected culture samples were stored under argon in containers, capped, sealed with Parafilm, and stored at –80 ˚C until further analysis. Samples (~5 g) of each ^{13}C labeled culture were reserved for further carotenoid and ^{13}C mass spectrometry analysis, and the remaining cells were combined for carotenoid extraction and preparatory separation for future mammalian metabolic studies.

HPLC Carotenoid Evaluation

Callus and suspension cultures were analyzed for carotenoid content by extracting ~0.25 g of cells using a carotenoid extraction and high pressure liquid chromatography-photodiode array (HPLC-PDA) analysis protocol previously published (11). General precautions for work with carotenoids were taken to prevent degradation and artifact formation during the extraction and analytical process (21). Carotenoids were identified and quantified by external standard curves using authentic LYC (isolated from DSM Redivivo Beadlets donated by DSM, Heerlen,

Netherlands), and PE and PF standards (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany).

Selection of the Highest LYC Accumulating Cell Line

Callus cultures of mutant high LYC lines, corresponding wild-type cell lines, and the 'VFNT cherry' cell line were evaluated for LYC accumulation during 3 trials with 2 reps per trial. Each rep consisted of 3 g of callus inoculum placed onto agar-solidified carotenoid production medium with or without CPTA. Mutant high LYC line CPTA-treated cultures were compared to corresponding CPTA-treated wild-type lines to verify that the mutation was responsible for LYC accumulation differences. The highest LYC accumulators and available corresponding wild-type callus cell lines were then used to initiate cell suspension cultures by transferring 2 g of friable callus cultures to liquid maintenance medium. After 8.5, 10.5, and 11 months of culture adaptation to liquid medium, cultures were transferred to carotenoid production medium for one 2-week adaptation period and subsequently transferred to fresh carotenoid production medium with or without CPTA for a 2-week carotenoid production growth period, and then harvested for subsequent analysis.

Comparison of hp-1 Carotenoid Production Grown with Sucrose or Glucose As the Carbohydrate Source.

Presently, the only economical [U]-^{13}C labeled carbohydrate for addition to cell culture media is glucose; however, standard tomato growth media utilizes sucrose. To determine the effect of medium carbohydrate source on harvest mass yield and carotenoid concentration, *hp-1* cell suspension cultures were grown in liquid carotenoid production media with either 30 g/L unlabeled sucrose or unlabeled glucose for a single, 14-day adaptation growth cycle, transferred

to fresh media, and treated with CPTA as specified above for a 14-day growth cycle, then harvested for analysis.

Production of ^{13}C-labeled LYC from hp-1 Tomato Cell Suspension Cultures

For LYC ^{13}C-biolabeling, 2 flasks of 80 mL liquid carotenoid production medium containing 30 g/L unlabeled glucose were inoculated with established cell suspension cultures for a media adaptation growth cycle. The contents of these two flasks were combined aseptically and 12 mL aliquots of the established cultures (4 mL of packed cells with 8 mL of spent media) were used to inoculate 3 flasks of fresh medium with 30 g/L [U]-^{13}C-glucose (Cambridge Isotope Laboratories, Inc., Andover, MA). The established cultures grown for the glucose adaptation growth cycle utilize glucose as an energy source, therefore it can be estimated that the resultant ^{13}C-glucose/^{12}C-glucose ratio in the fresh ^{13}C-containing media is greater than 10:1. Other ^{12}C-containing components in the media such as growth regulators and myoinositol accounting for less than 1% of the organic content in the media are likely minor contributors to carotenoid precursors. Cultures were grown in a previously described enclosed labeling chamber at 26 ˚C atop a rotary shaker (160 rpm) (22). To promote LYC accumulation, cultures were treated with CPTA on day 1, grown for 14 days, harvested, and stored for carotenoid and isotope enrichment analysis.

^{13}C-Carotenoid Mass Isotopomer Analysis

Biolabeled carotenoids were isolated from *hp-1* cell suspension cultures by a series of three HPLC separations. First, a previously optimized carotenoid extraction procedure for tomato cell suspension cultures was utilized (23). Carotenoid-rich extracts were first subfractionated to yield an LYC-rich fraction using a YMC C30 preparatory column (Waters, Milford, MA) with previously described conditions (11). Mobile phases in the carotenoid

containing eluate fractions were evaporated using a Savant AS160 Automatic Speedvac. This fraction was subfractionated a second time to remove coeluting contaminants using a Discovery C18 (25 cm x 4.6 mm, 5 μm) (Supelco, Sigma-Aldrich, St. Louis, MO) column following a previously published method (24). Eluted LYC was collected, and one third and final separation was performed on the same HPLC apparatus described using a high resolution HPLC method using a YMC C30 analytical column (4.6 x 25 cm, 3 μm) to obtain pure *all-E* LYC containing minimal coeluting carotenoids or contaminants (25). LYC from this purified fraction was then analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) using atmospheric pressure chemical ionization (APCI) at 2877 V, 400 °C, a nebulizer pressure of 60 psi, and dry temperature of 350 °C. Purified ^{13}C-LYC was dissolved in diethyl ether and injected onto a LC-MS/MS (LC-MSD-Trap-XCT plus, Agilent Technologies, Santa Clara, CA) equipped with a Zorbax Eclipse XDB C18 column (Agilent Technologies, Santa Clara, CA; 2.1 x 50 mm, 3.5 μm). Sensitivity of the LC-MS/MS used was 1-10 ng, and higher amounts were injected to facilitate the quantification needed for mass isotopomer analysis. To quantify the proportions of different isotopomers present in the ^{13}C-LYC peak, four injections of 1000 ng of ^{13}C-LYC in 40 mL of diethyl ether were made, and data acquisition parameters were set to collect signal intensities for LYC masses on positive ion mode ranging from *m/z* 572.4-577.4 (M + 35 to M + 40) for injection 1, *m/z* 567.4-572.4 (M + 30 to M + 35) for injection 2, *m/z* 562.4-567.4 for injection 3 (M + 25 to M + 30), and *m/z* 557.4-562.4 for injection 4 (M + 20 to M + 25). Mass chromatogram peak areas generated from the resulting runs were normalized on the basis of repeated *m/z* channels analyzed between runs and were then quantified. Mass chromatogram peak areas for masses below 557.4 were not quantifiable; therefore, the effective range of masses evaluated was from *m/z* 557.4-577.4 (M+20 to M+40). Relative signal

intensities for carotenoid mass isotopomers were compared, and the ^{13}C-enrichment profile was quantitatively determined using spectral peak areas.

Statistical Analysis

Statistically significant treatment and cell line differences for >2 groups were determined by analysis of variance (ANOVA), and when the normality and homogeneity of variance assumptions of ANOVA were met, the posthoc Tukey's studentized range test was used to identify specific differences. When the assumptions of ANOVA were not met, the nonparametric Wilcoxon and Kruskal-Wallis tests were used to detect significant differences. When only two treatments were being compared, the two-sample *t* test was used, and when the normality and homogeneity of variance assumptions of the *t* test were met, the equal variances *t* test was used to determine significant differences. If both the normality and homogeneity of variance assumptions of the two-sample *t* test were not met, the Wilcoxon two-sample test was used. If only the homogeneity of variance assumption of the two-sample *t* test was violated, then Cochran modified *t* test for unequal group variance was used. All statistical analyses were performed using the statistical analysis software SAS, versions 7.1 and 9.2 (SAS Institute, Inc., Cary, NC). Averages are presented with the standard error of the mean whenever possible.

Results and Discussion

1. gh and Wild-Type Tomato Cell Cultures

1.1 Callus and Suspension Culture Initiation.

Eighty-percent of the seeds from wild-type ($^{+}/^{+}$ or $^{+}/gh$) tomato germinated within 9 days and of those, 10% were positively identified as *gh* mutants by the presence of variegated true leaves. Callus growth was observed within 16 days on the surface of the *gh* stem and leaf

explants and within 30 days on *gh* flower bud explants. Callus growth on wild-type explants was observed within 41 days on leaves and within 20 days on flower buds.

1.2 Carotenoid Accumulation by Genotype and Explant Source.

Wild-type and *gh* callus cultures were sampled and analyzed for carotenoid concentrations. The *gh* flower bud-derived callus culture accumulated the greatest amount of total carotenoids with PE being the major carotenoid (5.6 ± 1.8 μg/g callus) and PF, LYC, and β−carotene (Table 3.1) in lower concentrations. Both leaf and flower bud-derived *gh* callus cultures had greater overall carotenoid accumulation than the wild-type callus cultures, though the wild-type-derived callus cultures had greater β-carotene concentrations than the *gh* callus cultures. The phenomenon where one *in vitro* vegetative tissue differs biochemically from another has been seen before (26). Previous studies of *in vitro* 'VFNT cherry' tomato sepals, the vegetative tissue that forms a protective layer around the flower, indicated that this tissue has the potential to undergo ripening processes including LYC accumulation, swelling, and production of ethylene and its precursor, without manipulation of the media growth regulators or attachment to fruit. The results in this article support the finding that sepal tissue of the flower bud may retain a potential to change the developmental fate from vegetative tissue to fruit-like tissue, allowing ripening processes, which include increased carotenogenesis (26). In that same study, it was observed that another tomato cultivar, 'Ailsa Craig', did not exhibit the same sepal ripening process, suggesting that the involvement of a genetic component for this retained the ripening potential in sepal tissue (26). *In vitro* explant-specific secondary metabolite profiles from callus cultures of red clover for isoflavones and of strawberry for anthocyanins have also previously been observed (12, 27). Flower bud-derived *gh* callus exhibits more ripening processes than leaf-derived calluses, however, the wild-type callus types did not show this same trend,

suggesting that there may be a genotype effect on carotenoid accumulation in differentially derived callus tissues.

1.3 Treatment with Herbicides.

A brief investigation on the impact of CPTA and norflurazon on carotenoid accumulation in *gh* and wild-type cell suspension cultures grown in carotenoid production media (one trial/herbicide treatment, n = 2-3 reps/treatment) showed that the *gh* cell line yielded greater harvest mass than the wild-type cell line, regardless of herbicide treatment. In the CPTA and norflurazon experiments, *gh* cell suspension cultures yielded almost 3 times more cell mass than wild-type cultures (363 vs 95 and 347 vs 90 g cells/L culture, respectively). Counter to the initial hypotheses for this study, neither of the herbicides had a substantial impact on PE or PF accumulation in *gh* cultures; however, herbicide treatments did impact carotenoid accumulation profiles in wild-type cell suspension cultures (Tables 2 and 3). These results suggest that these bleaching herbicides may cause greater total carotenoid accumulation in wild-type cultures by causing a lack of downstream carotenoid products necessary for feedback regulation of carotenogenesis. Since the *gh* cell line already largely lacks downstream carotenoids, the bleaching herbicides did not confer any additional stimulation of PSY or inhibition of PDS. In a previous study, mRNA levels of carotenogenic enzymes in *gh* tomato fruit were similar to the levels in wild-type fruit, suggesting that a lack of downstream carotenoid products may not increase the transcription of these enzymes (20). It may be possible that the activity of the PSY enzyme is increased in the *gh* cell line compared to that in the wild-type cell line. CPTA-treated wild-type cultures did have increased LYC accumulation compared to that in the control wild-type and CPTA-treated or control *gh* cultures (Table 3.2). Norflurazon-treated wild-type cultures, however, did not produce as much PE as the *gh* cultures (Table 3.3). On the basis of

these results indicating that untreated *gh* cell suspension cultures accumulate and yield the greatest amount of PE, herbicide treatment of the *gh* cell line was not pursued further. All additional *gh* experiments focused on the untreated *gh* cell line.

1.4 Comparison of gh Carotenoid Production from Cultures Grown in Different Media Formulations.

Three trials were performed to evaluate *gh* cell suspension culture carotenoid production and cell mass accumulation from cultures grown in carotenoid production versus maintenance medium. Cultures grown in the carotenoid production media produced significantly greater cell mass (211 ± 7.2 g/L) than the maintenance media-grown cultures (150 ± 4.1 g/L) (p=0.0017). PE yield was also greater in carotenoid production media (3458 ± 612 μg/L) than from maintenance media (489 ± 84 μg/L; p = 0.04). On the other hand, PF yield was not significantly different between media conditions (maintenance media: 27.8 ± 2.2 μg/L; carotenoid production media: 78.7 ± 16.8 μg/L; p = 0.09). This experiment confirmed the hypothesis that carotenoid accumulation would be greater in cultures grown on the carotenoid production media formulation. The PE yield from *gh* cultures grown on carotenoid production media is greater than that of 'VFNT cherry' tomato cell suspension cultures treated with norflurazon (2060 μg/L), a previously established suspension culture system for PE production (10). On the basis of these findings, it can be concluded that *gh* cell suspension cultures are a superior source of PE.

2. High LYC Lines

2.1 Callus Culture Initiation.

Tomato flower buds from greenhouse-grown high LYC tomato plants and their corresponding wild-type plants were explanted to agar-solidified callus induction media. Callus was observed on all explants within a 1-4 wk growth period, and proliferating, friable callus was

obtained within 2-3 growth cycles of the 'Ailsa Craig' wild-type and *hp-1*, 'Manapal' wild-type and *hp-2*^{dg}, 'Suncoast' *B^og*^{c}, *L. pimpinellifolium*, and 'VFNT cherry'. 'Ailsa Craig' *B^og* did not adapt well to the callus induction media and was not included in subsequent studies.

2.2 Selection of Highest LYC Accumulator

Three trials examining several high LYC lines, corresponding wild-type lines, and the 'VFNT cherry' line callus cultures for LYC accumulation were harvested and analyzed. Harvest yields of CPTA and untreated 'VFNT cherry' callus cultures were significantly greater than those in other CPTA or untreated cultures, respectively (Table 3.4). Carotenoid concentrations were greatly increased when CPTA was added to the agar-solidified carotenoid production medium (Table 3.5). In untreated cultures, there was no cultivar or genotype effect on combined PE, PF, β-carotene, and LYC accumulation. Alternatively, when CPTA was present in the media the *hp-1* cell line had a significantly greater combined acyclic carotenoid (PE, PF, and LYC) concentration (45.8 ± 13.1 μg/g cells) than the other cell lines including its corresponding wild-type (27.2 ± 4.5 μg/g cells), suggesting that the *hp-1* genotype is responsible for the enhanced carotenoid accumulation. All cell cultures treated with CPTA had only trace amounts of β-carotene (data not shown). The two highest carotenoid accumulators, 'Ailsa Craig' *hp-1* and 'Manapal' *hp-2* (45.8 ± 13.1 and 31.3 ± 7.1 μg/g cells, respectively), in callus culture were thus selected along with their corresponding wild-types to be transferred to solution culture and evaluated for LYC production in comparison to previously established 'VFNT cherry' cell suspension cultures.

Three trials were initially performed to evaluate 'Ailsa Craig' *hp-1* and wild-type, 'Manapal' *hp-2* and wild-type, and 'VFNT cherry' cell suspension cultures for LYC production. Over the period of the three trials, substantial changes in LYC concentration and yield were

observed (Figure 3.2 A & B). Some tomato cell lines adapted to the carotenoid production liquid medium, while other cell lines performed more poorly over the 2.5-month span in which the three trials were undertaken. The changes in harvest mass and LYC concentrations over the 2.5-month trial period were substantial, but this is common during the establishment of *in vitro* plant cell lines. Bourgaud and colleagues discussed this phenomenon in callus cultures previously, suggesting that in many cases erratic production of plant secondary metabolites *in vitro* can be linked to genetic instability (17). Genetic instability, according to their assessment, is often under-reported in publications but has been observed to take up to 16 subculture cycles, or 48 wk, to dissipate. One practical suggestion given by these authors was to use cell growth reproducibility, a polygenic characteristic, over three consecutive growth cycles as a marker for genetic stability (17). Although these authors discussed the appearance of this phenomenon in callus cultures, from the current studies, it appears that genetic instability is apparent in cell suspension cultures and that adaptation time can be lengthy. For this reason, tomato cell line selection was based on LYC yields obtained from the final trial completed 11 months postcell suspension culture initiation. Eleven months postinitiation of cell suspension cultures, 'Ailsa Craig' *hp-1* yielded the greatest amount of LYC (7.58 mg/L culture) and was 1.6-fold greater than the 'VFNT cherry' LYC yield (2.97 mg/L culture) followed by 'Ailsa Craig' wild-type (5.47 mg/L culture) (Figure 3.2B). 'VFNT cherry' cell suspension cultures produced the highest concentrations of PE with CPTA-treatment (5.22 μg/g cells) and were also the greatest accumulators of PE when untreated (0.70 μg/g cells), compared to the other cell lines (Table 3.6). 'Ailsa Craig' *hp-1* also yielded the greatest cell mass (199 g/L) at the 11-month harvest. The high cell mass production combined with high concentrations of LYC led to the selection of the 'Ailsa Craig' *hp-1* for subsequent herbicide treatment and ^{13}C-biolabeling studies. To

ascertain genetic stability, the three subsequent herbicide trials were run consecutively, per the recommendations of Bourgaud et al. (17).

2.3 Treatment with Herbicides

Three consecutive trials with three repetitions per trial were harvested to simultaneously evaluate the genetic stability of and carotenoid accumulation in *hp-1* cell suspension cultures in response to different herbicide treatments. Within the control, CPTA, and norflurazon treatments, there were no significant harvest mass yield differences between trials according to the Kruskal-Wallis test (average yields were 218 ± 42 g/L, 156 ± 16 g/L, and 220 ± 40 g/L, respectively). However, there was a significant difference within the CPTA and norflurazon combination treatment group between trials ($\alpha = 0.05$) between the first (214 ± 33 g/L) and third (119 ± 3 g/L) trials, but neither was different from the second trial (183 ± 3 g/L) according to Tukey's studentized range test. Although there were differences between the harvest yields of the CPTA and norflurazon combination treatment between trials, the remainder of the treatments had statistically consistent harvest yields and can be viewed, on the whole, as genetically stable. When harvest yields were compared between treatments for all trials, there were no significant differences, including the CPTA and norflurazon combination treatment (average 172 ± 28 g/L; other treatment averages given above). Carotenoid yields were analyzed for each trial (Figure 3.3). There were no significant differences in individual carotenoid concentrations by herbicide treatment; however, total carotenoid yields in herbicide-treated cultures were significantly greater than the control cultures ($\alpha = 0.05$) (Figure 3.3). The PE, PF, and LYC accumulation patterns were similar to, but overall greater than, the accumulation pattern previously seen in 'VFNT cherry' tomato cell suspension cultures treated with these herbicides (10) (3.6 - 5.2 mg/L culture vs 2.3-3.3 mg/L culture, respectively) (10). This result suggests that *hp-1* tomato cell

suspension culture is superior to the previously established 'VFNT cherry' cell suspension culture system. The untreated *gh* tomato cell line, when grown on carotenoid production media, also produced substantially more PE than previously published values for the norflurazon-treated 'VFNT cherry' line, but had a lesser PE accumulation than the norflurazon-treated *hp-1* cell line. On the basis of these findings, the *hp-1* cell line treated with CPTA was selected for ^{13}C-LYC labeling.

3. Tomato Cell Suspension Culture ^{13}C-Carotenoid Biolabeling

3.1 Comparison of Carotenoid Production in hp-1 Cell Suspension Cultures Grown with Sucrose or Glucose as the Carbohydrate Source.

Carbohydrate source in the tomato cell culture liquid media did not have a significant impact on harvest mass yield (glucose, 199 ± 19 g/L; sucrose, 206 ± 2 g/L). Neither total carotenoid yield (glucose, 6.22 ± 0.34 mg/L; sucrose, 5.81 ± 1.17 mg/L) nor individual carotenoid proportions significantly varied by carbohydrate source (data not shown).

3.2 ^{13}C-labeled carotenoid yields from hp-1 cell cultures.

Harvest mass yields for *hp-1* cell cultures grown with uniformly labeled ^{13}C-glucose as the primary carbon source (198 ± 3.6 g/L) were very similar to yields from cultures grown with unlabeled glucose. Carotenoid yields were, however, slightly lower than those seen in the herbicide and carbohydrate source experiments (PE, 0.313 ± 0.013; PF, 0.12 ± 0.01; LYC, 2.45 ± 0.11). After three HPLC purification steps of the plant cell culture carotenoid extract, purified LYC was obtained (Figure 3.4). LYC was analyzed and purified for mass isotopomer analysis, and the results are shown in Figure 3.5. *In vitro* labeling does not yield 100% uniformly labeled LYC but rather a range of isotopomers (m/z=557-577), which represent the M + 20 to + 40 isotopomer masses. The uniformly labeled isotopomer (m/z=577, M + 40) was the most

prominent isotopomer constituent (45%) of the labeled LYC fraction, and 88% of the LYC molecules were present as M + 35 to + 40 masses. Production of a distribution of β-carotene and LYC mass isotopomers has been previously reported in other biological systems (28-30). By providing uniformly labeled glucose as the primary source of carbon in this *in vitro* system, the labeled LYC produced from this experiment had a much greater proportion of uniformly labeled (*m/z* = 577) LYC than what has been seen in these other reports of deuterium enriched β-carotene and LYC. In this experiment, the unenriched isotopomer (*m/z* = 537) was not detected in purified ^{13}C-LYC isolated from *hp-1* cell cultures. In conclusion, a strategic screening and selection process for carotenoid accumulation in multiple *in vitro* tomato cell lines noted for their elevated *in vivo* carotenoid accumulation was used to develop a production system for isotopically labeled carotenoids for utilization in human and animal metabolic tracing studies. To the authors' knowledge, this is the first report of the production of highly enriched ^{13}C-carotenoids from a plant cell culture system for dietary research.

Future work should focus on stimulating PF accumulation in tomato cell suspension cultures. Additionally, even higher uniform enrichments of tomato cell line-derived ^{13}C-carotenoids may be achieved by sequential growth cycles in ^{13}C-glucose containing media. Future research will focus on the utilization of this highly enriched ^{13}C-LYC as well as the production of ^{13}C-PE and PF from norflurazon-treated *hp-1* cell suspension cultures for mammalian cell culture and human studies. The advances made in this work provide the opportunity for greater elucidation of mammalian carotenoid metabolism as well as underlying mechanisms of bioactivity.

Abbreviations Used:

LYC, lycopene; PE, phytoene; PF, phytofluene; CPTA, 2-(4-chlorophenyl-thio)triethylamine; PTOX, plastid terminal oxidase; HPLC-PDA, high pressure liquid chromatography-photodiode array; BHT, butylated hydroxytoluene

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Tables

Table 3.1. Carotenoid Concentrations from *gh* and Wild-Type- Callus Cultures (One Trial, *n* = 5 Replicates)[a].

		Carotenoid concentrations (μg/g callus) ± SEM			
Ghost Cell Line Genotype	Explant Tissue Type	Phytoene	Phytofluene	Lycopene	β–carotene
wild-type	Leaf	n/d	n/d	0.01 ± 0.00	0.06 ± 0.01
	Flower bud	n/d	n/d	0.01 ± 0.00	0.06 ± 0.01
gh	Leaf	1.41 ± 0.24	trace	0.02 ± 0.01	0.04± 0.00
	Flower bud	5.59 ± 1.82	0.16 ± 0.04	0.03 ± 0.00	0.03 ± 0.01

[a]Carotenoid concentrations represent the average of five samples ± the standard error of the mean (SEM).

Table 3.2. Carotenoid Concentrations from Control *gh* and Wild-Type Cell Suspension Cultures or Treated with CPTA for the 14 d growth cycle.[a]

		Carotenoid concentrations (μg/g cells)			
GH Cell Line Phenotype	Herbicide Treatment	Phytoene	Phytofluene	Lycopene	β−carotene
wild-type	CPTA	0.10	0.10	4.23	0.23
	Control	n/d	trace	0.04	0.87
gh	CPTA	25.10	0.65	0.33	0.02
	Control	30.87	0.75	0.19	0.17

[a]Carotenoid concentrations represent the average of two samples analyzed from one trial.

Table 3.3. Carotenoid Concentrations from Control *gh* and Wild-Type Cell Suspension Cultures Grown in Carotenoid Production Media for 14 Days and Treated with Norflurazon on Day 7.[a]

		Carotenoid concentrations (μg/g cells)			
GH Cell Line Genotype	Herbicide Treatment	Phytoene	Phytofluene	Lycopene	β–carotene
wild-type	Norflurazon	9.53	0.65	0.05	0.41
	Control	0.33	0.06	0.06	1.07
gh	Norflurazon	14.08	0.18	0.11	0.15
	Control	15.03	0.33	0.05	0.17

[a]Carotenoid concentrations represent the average of two samples analyzed from one trial.

Table 3.4. Harvest Yields of Callus Cultures of High Lycopene Tomato Cell Lines and Available Wild-Type Cultures Grown on Agar-Solidified Carotenoid Production Medium with or without CPTA.[a]

	Harvest Yield (g callus/3 g callus inoculum) ± SEM	
Tomato Cell Line	Control Treatment	CPTA Treatment
'VFNT cherry'	14.47 ± 1.79^{A}	13.23 ± 1.28^{A}
'Manapal' wild-type	6.65 ± 0.35^{B}	5.85 ± 0.25^{B}
'Manapal' hp-2	9.16 ± 1.42^{B}	8.26 ± 1.68^{B}
'Ailsa Craig' wild-type	7.35 ± 0.71^{B}	6.27 ± 0.35^{B}
'Ailsa Craig' hp-1	6.73 ± 0.23^{B}	5.51 ± 0.21^{B}
L. pimpinellifolium	8.44 ± 1.84^{B}	8.72 ± 0.77^{B}
'Suncoast' B^og	7.99 ± 0.35^{B}	7.95 ± 0.31^{B}

[a]Values represent the average mass yield of three trials with two reps per trial, where one rep was one container of agar-solidified medium inoculated with 3 g callus. Statistically different yields within the treatment groups were detected by analysis of variance (α=0.05), and are denoted by different superscript letters.

Table 3.5. Carotenoid Concentrations of Control-Treated and CPTA-Treated Tomato Callus Cultures Grown on Agar-Solidified Carotenoid Production Medium.[a]

	Carotenoid Concentration (µg/g cells)					
	Phytoene		**Phytofluene**		**Lycopene**	
	Control	**CPTA**	**Control**	**CPTA**	**Control**	**CPTA**
'VFNT' cherry	0.48 ± 0.22	0.89 ± 0.09	0.35 ± 0.16	0.90 ± 0.07	1.16 ± 0.56	10.90 ± 3.72
'Ailsa Craig'						
hp-1[b]	0.44 ± 0.07	2.67 ± 1.02	0.32 ± 0.05	2.12 ± 0.50	0.62 ± 0.22	40.98±11.56
'Ailsa Craig' WT	0.32 ± 0.14	1.12 ± 0.15	0.23 ± 0.10	0.95 ± 0.12	0.41 ± 0.18	25.12 ± 4.28
L.						
pimpinellifolium	0.54 ± 0.10	0.96 ± 0.07	0.39 ± 0.07	0.70 ± 0.10	1.29 ± 0.52	15.40 ± 2.84
Manapal' *hp-2*	0.59 ± 0.15	1.59 ± 0.34	0.43 ± 0.11	1.73 ± 0.38	1.49 ± 0.61	28.01 ± 6.41
'Manapal' WT	0.45 ± 0.20	0.85 ± 0.08	0.32 ± 0.15	0.74 ± 0.07	0.95 ± 0.51	13.31 ± 1.10
Suncoast' B^og	0.54 ± 0.18	0.71 ± 0.01	0.39 ± 0.13	0.52 ± 0.00	0.91 ± 0.37	7.90 ± 1.56

[a]Average values of three trials with two replicate analyses per trial and SEM are presented.

[b]The CPTA-treated 'Ailsa Craig' *hp-1* cell line had a significantly greater total carotenoid (phytoene + phytofluene + lycopene) concentration than all other treatments and cell lines as detected by analysis of variance ($\alpha = 0.05$).

Table 3.6. Carotenoid Concentrations and Harvest Yields from Tomato Cell Suspension Cultures Harvested 11 Months Postcell Suspension Culture Initiation.[a]

	Carotenoid Concentration (μg/g cells)						Harvest Yield (g cells/L culture)	
	Phytoene		Phytofluene		Lycopene			
	Control	CPTA	Control	CPTA	Control	CPTA	Control	CPTA
'VFNT' cherry	0.70	5.22	n/d	0.57	0.01	21.61	178	137
'Ailsa Craig' hp-1	0.50	1.69	n/d	0.20	n/d	38.09	270	199
'Ailsa Craig' WT	0.01	0.62	n/d	0.09	n/d	39.52	192	138
'Manapal' hp-2	0.01	0.73	n/d	0.09	n/d	27.55	131	84
'Manapal' WT	0.01	0.17	n/d	0.03	n/d	10.63	168	147

[a]Cultures were treated with or without CPTA and harvested after one 14 d growth period. Each value represents the average of two samples. Samples with either no signal for a carotenoid or a signal below accurate quantification are denoted at not detectable (n/d).

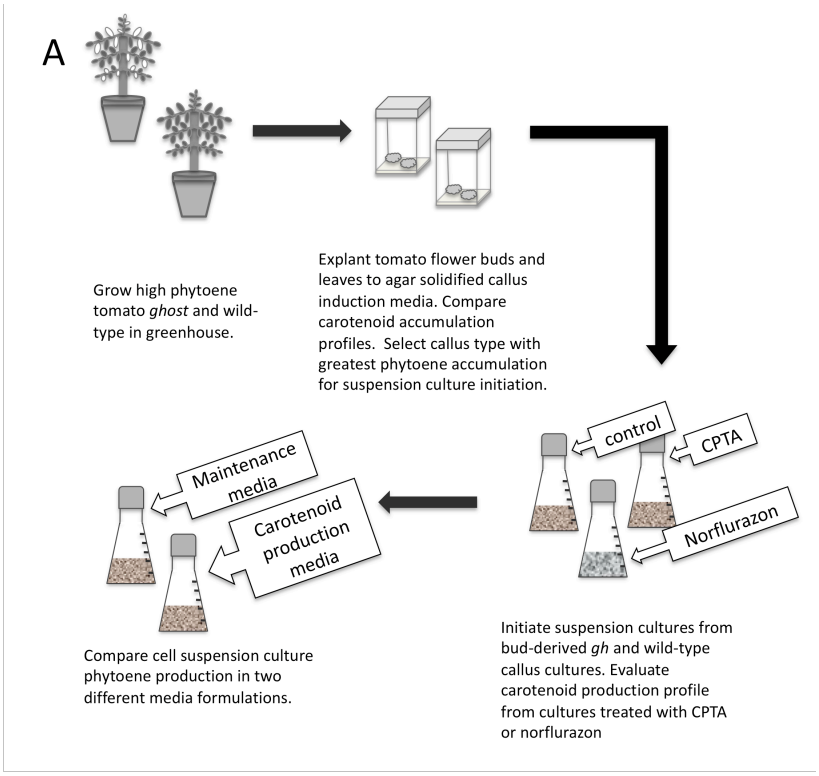

Figure 3.1. Simplified study design for derivation and selection of tomato cell lines for A. phytoene production from the *ghost* tomato cell line, and B. lycopene, phytoene, and phytofluene production from a high lycopene tomato cell line.

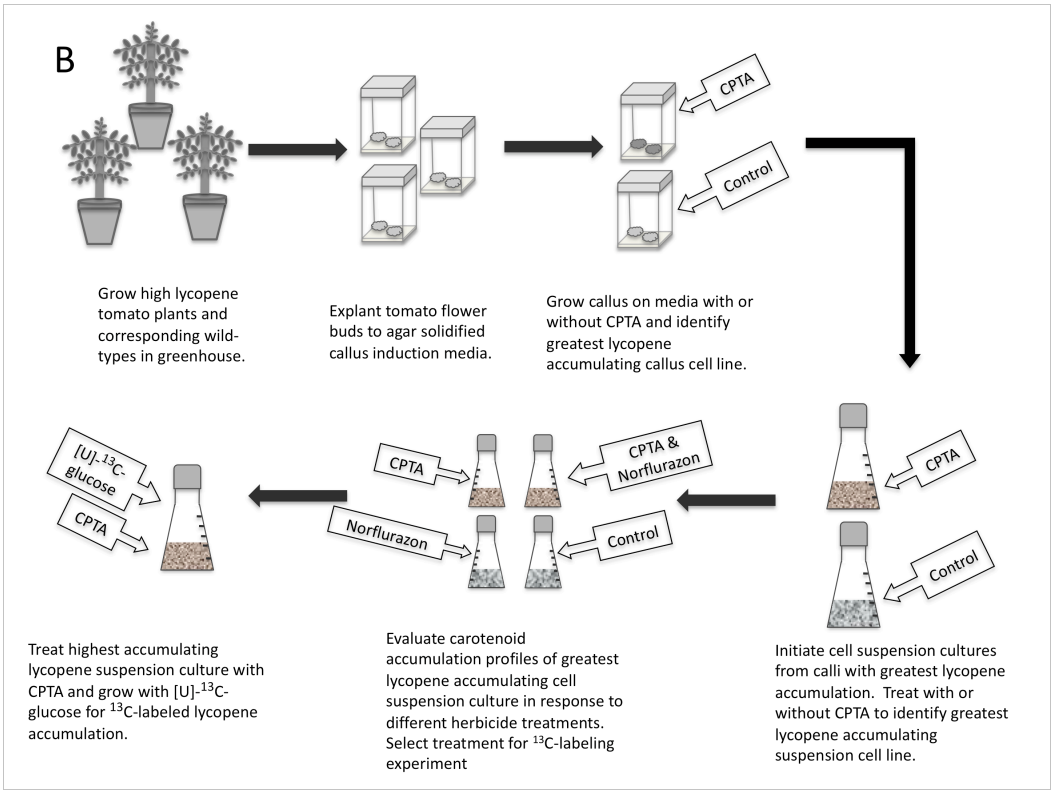

Figure 3.1. (cont.)

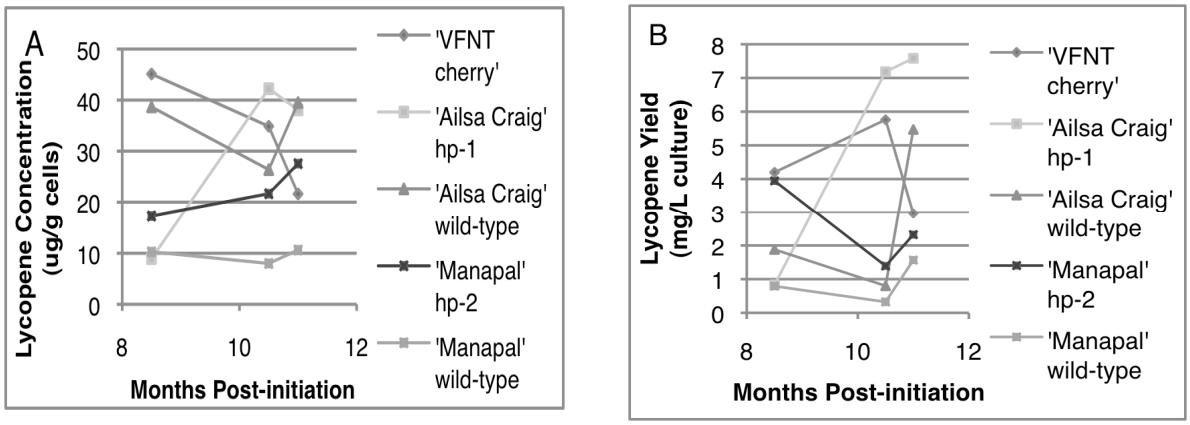

Figure 3.2. Lycopene concentrations (A) and yields (B) from different tomato cell lines suspension cultures treated with CPTA for a 14 day growth cycle and harvested approximately 8.5, 10.5, or 11 months post-initiation of cell suspension cultures from callus cultures. Each point represents the average of two samples.

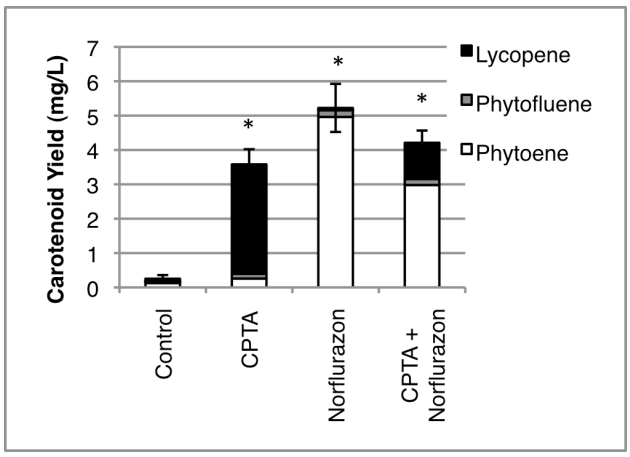

Figure 3.3. Combined carotenoid (LYC, PF, and PE) yields from *hp-1* tomato cell suspension cultures treated with different herbicides (n = 3 trials). Error bars represent SEM of combined carotenoids. Significantly different combined carotenoid yields (α = 0.05) detected by Tukey's studentized range test compared to control are noted with an asterisk (*).

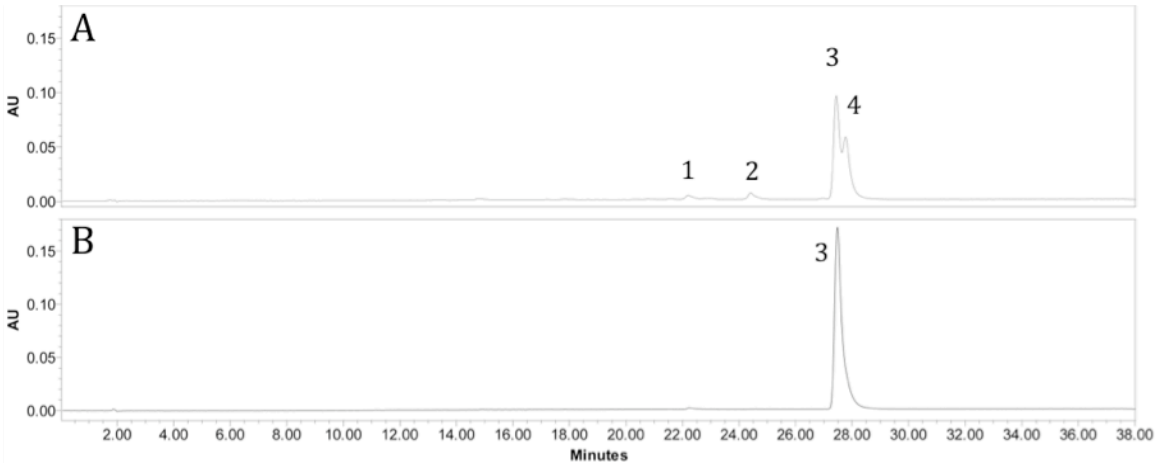

Figure 3.4. HPLC chromatograms (λ = 472 nm) of (A) LYC analytical standard with *all-E* (peak 3) and 5-*Z* (peak 4) lycopene isomers eluting at 28 min with other preceding *Z*-isomers (RT = 22.5 and 24.5 min; peaks 1 & 2) and (B) purified ^{13}C-*all-E* LYC from a *hp-1* tomato cell suspension culture.

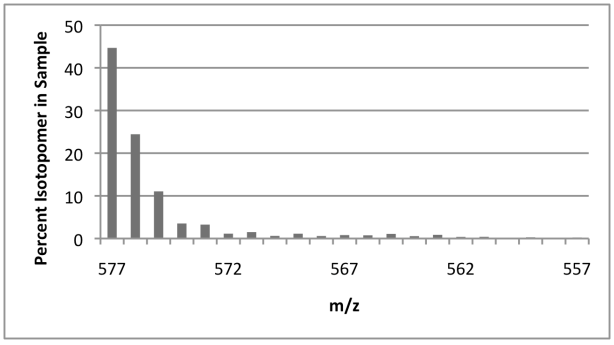

Figure 3.5. Mass isotopomers present in purified ^{13}C-lycopene from *hp-1* tomato cell culture treated with CPTA. Percent isotopomer in sample is calculated from mass chromatogram peak signal areas obtained from four analytical injections of ^{13}C-lycopene (1000 ng each in 40 µL diethyl ether); each injection yielded peak area signals for 6 masses selected, and sequential injections were normalized. Signals for < m/z = 557 were not quantifiable. *In vitro* labeling yields 45% uniformly labeled (m/z = 577) ^{13}C-lycopene and a distribution of less enriched isotopomers (m/z= 557-576).

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CHAPTER 4:
Relative bioavailability of phytoene and lycopene and bioaccumulation of tomato carotenoids in tomato powder-fed Mongolian gerbils (*Meriones unguiculatus*)

Abstract

Tomato carotenoid consumption is associated with decreased risk of chronic disease development, including cardiovascular disease and prostate cancer. Although lycopene (LYC) is often credited as the single bioactive, tomatoes provide a complex milieu of nutrients and bioactives including the carotenoids phytoene (PE), phytofluene (PF), ζ-carotene (ZC), and β-carotene (BC). The objective of this study was to determine the biodistribution of tomato carotenoids from a tomato containing diet as well as compare the relative bioavailability of PE and LYC in oil in a relevant animal model, the Mongolian gerbil. Tomato carotenoids accumulated in tissues and serum collected from gerbils fed 10% tomato powder diets for 26 days and then fed a control diet for 2 days, with LYC being the primary carotenoid in serum, liver, spleen, lungs, testes, and prostate-seminal vesicle complex. PF was the most concentrated carotenoid in the adrenal glands, while PE was the most concentrated carotenoid in the gonadal adipose. LYC, PF, and ZC were detectable in all tissues evaluated, while BC was not detected in the testes, gonadal adipose, prostate-seminal vesicle complex, lung, or spleen, and PE was not detected in the prostate-seminal vesicle complex or the testes. Kinetic dosing of tomato powder pre-fed gerbils with PE or LYC in oil showed that PE was more readily absorbed and biodistributed to the tissues, causing significant increases at 6, 12, and 24 hr after dosing in PE concentrations in serum (570%, 340%, and 650 %, respectively), liver (6 hr, 81%; 24 hr, 109%), and the spleen (1722%, 1400%, and 1633%, respectively) . LYC dosing also significantly increased serum *Z*-LYC isomer concentrations at 6, 12, and 24 hours (90%, 60%, and 30%) and *all-E* LYC at 6 hours post-dosing (40%), but did not lead to significant increases in tissue LYC. This study suggests that the colorless carotenoid, PE, is more bioavailable than LYC in tomato

powder pre-fed gerbils, and that the gerbil is a suitable model for studying tomato carotenoid absorption and biodistribution.

Introduction

Epidemiological evidence suggests that tomato product consumption can decrease the risk of two widespread chronic diseases: cardiovascular disease and prostate cancer *(1, 2)*. Tomatoes contain a variety of nutrients and constituents that are part of a healthy diet such as folate, vitamin E, vitamin C, potassium, pro-vitamin A carotenoids, and fiber *(3)*. In addition to these nutrients, tomatoes are a rich source of carotenoids including lycopene (LYC), neurosporene, γ-carotene, β-carotene (BC), ζ-carotene (ZC), phytoene (PE), and phytofluene (PF). Carotenoids are colorful tetraterpene molecules produced by plants, fungi, and bacteria, and were only recently discovered to be synthesized in animals (the pea aphid) *(4)*, albeit exceptionally rare. Tomato carotenoids have a variety of bioactive functions that may relate to decreased chronic disease risk. LYC has been most widely investigated as a potent antioxidant, inducer of phase II enzymes, supressor of cell proliferation, and upregulator of gap-junction communication *(5)*. The colorless tomato carotenoids, PE and PF, similarly to LYC, are antioxidants and inhibit LDL oxidation *in vitro (6)*. Further, when mouse embryonic fibroblasts (NIH3T3) were engineered to produce PE, the cells were protected from oxidative stress as well as malignant transformation induced by oncogene transfection *(7)*. When rats were fed either PF, LYC, or tomato powder for 4 d, their circulating androgens were depressed *(8)*. A small study of human autopsy tissue samples indicated that LYC, PE, PF, and ZC are accumulated at ng/g concentrations in liver, lung, and prostate tissues *(9)*. Previous studies of tomato carotenoid bioaccumulation in rats suggested that PE, PF, LYC, and ZC are differentially accumulated in tissues and tissue carotenoid profiles did not directly mimic the profile of carotenoids provided in the diet.

Notably, PF was the predominant carotenoid in the liver, and PE and PF were the major carotenoids in the adrenal glands as well as serum *(10)*. LYC is known to be the predominant carotenoid accumulated in both the human prostate as well as the rat, a commonly used animal model of prostate cancer. However, PE, PF, and ZC also substantially accumulated in the rat prostate at 23, 36, and 23% of the level of LYC, respectively and at 12, 54, and 50% that of LYC in the human prostate *(9-11)*.

The Mongolian gerbil has physiological similarities to humans for carotenoid, cholesterol, and lipid absorption and is thus an excellent model for carotenoid absorption and metabolism studies *(12)*. When gerbils were directly compared to F344 rats, BALB/c mice, and nude mice to evaluate LYC absorption and accumulation, it was found that gerbils most readily absorbed LYC, attaining plasma concentrations similar to those of humans with LYC dosing every other day (20 mg/kg BW•2d) accumulated the most hepatic LYC. This led to the conclusion that gerbils are the most suitable rodent species, of those evaluated, for studying the *in vivo* effects of LYC *(13)*.

Previously, the gerbil was utilized to evaluate relative bioavailability of LYC from different sources: 60 μg/d of LYC was delivered for 21 days *via* purified oral doses of LYC, freeze-dried tomato paste or red carrot, or from red carrot or tomato paste extracts in oil *(14)*. While LYC from a tomato product was evaluated, the uptake of PE, PF, and ZC was not. Furthermore, in the gerbil only serum, liver, adrenal, and testes have been studied. For a more complete picture of carotenoid biodistribution in this animal model, LYC, PE, PF, ZC, and BC accumulation in various tissues in response to tomato powder feeding should be evaluated *(13, 14)*. Tissue carotenoid concentrations in an appropriate model will serve as a reference for future *in vitro* and *in vivo* study designs to examine the absorption, metabolism, and bioactivity of

tomato carotenoids. Currently, nothing is known about the relative uptake and biodistribution kinetics of a single oral PE or LYC dose in gerbil tissues. A direct comparison of the absorption and biodistribution of PE and LYC will help to elucidate differences in absorption and biodistribution patterns of these two carotenoids.

In this current study, tomato carotenoid bioaccumulation was assessed in the serum, liver, spleen, adrenal glands, lungs, adipose, testes, and the prostate-seminal vesicle complex in gerbils fed 10% tomato powder diet for 26 days. In addition, gerbils pre-fed tomato powder-containing diets were dosed with either cottonseed oil, PE in oil, or LYC in oil to compare the differential absorption and biodistribution of these carotenoids over a 24 hour time course.

Materials and Methods

Animals and Experimental Design

This animal protocol was approved by the University of Illinois Institutional Animal Care and Use Committee and followed all necessary protocols to ensure humane treatment of the animals. Male, 40-day old Mongolian gerbils (38.12 ± 0.36 g BW) (Charles River Laboratories) (N = 56) were fed a pelleted chow diet (Teklad 8640, Harlan Laboratories, Madison, WI) for 2 days and were then fed a control, semi-purified, powdered diet published by Mills, et al. for 2 days *(15)* and were then randomized to either a control diet group (n = 7) or a 10% tomato powder diet group (n = 49) (Table 4.1, Figure 4.1). The tomato powder diet provided *all-E*-LYC (213.6 mg/kg), *Z*-LYC isomers (82.7 mg/kg), PE (11.7 mg/kg), PF (3.7 g/kg), ZC (5.5 g/kg), and BC (3.3 g/kg) (Futureceuticals, Momence, IL) (Figure 4.2), while the control diet provided 0.3 mg PE/kg diet andnone of the other carotenoids were detected. The tomato powder diet was balanced for macronutrients and fiber, with respect to the control diet (Table 4.1). Over the next 9 days, gerbils started their assigned study diets. Throughout the study, animals were weighed

and fed fresh powdered diet (15 g) every 2 days. Animals were assigned to dosing groups as follows: the tomato powder diet-fed animals were assigned to receive either an oral gavage of PE in cottonseed oil (TPPE group; $n = 21$), LYC in cottonseed oil (TPLY group; $n = 21$), or cottonseed oil alone (TPCO group; $n = 7$). The control-diet fed animals were assigned to receive the cottonseed oil dose (CCO group) ($n = 7$) (Figure 1). After 26 days on the study diet, all tomato powder fed animals were switched to a control diet for 2 days before carotenoid dosing to assure that there would not be any competition between dosed carotenoids and previously-fed dietary carotenoids for absorption. On the basis of the dosing assignment, gerbils were placed under isoflurane anesthesia and were provided with either 1.31 ± 0.05 mg LYC in 206 ± 3 μL cottonseed oil, 1.00 ± 0.01 mg PE in 202 ± 3 μL cottonseed oil, or 206 ± 4 μL cottonseed oil only (TPCO group). TPPE and TPLY animals were sacrificed at either 6, 12, or 24 hours after dosing ($n = 7$/timepoint) and TPCO animals were gavaged with cottonseed oil were sacrificed 6 hours post-dosing (Figure 4.1). At the time of sacrifice, gerbils were anesthetized with CO_2 and blood was collected by cardiac puncture; then animals were euthanized by CO_2 asphyxiation. The liver, adrenal gland, gonadal adipose, spleen, lung, testes, and the prostate-seminal vesicle complex tissues were collected, weighed, and snap frozen in liquid nitrogen, then stored at -80 °C for further carotenoid analysis.

Carotenoid analysis of diet composition

Tomato powder and control diets were analyzed by first extracting 0.025 g diet using a previously-established protocol *(16)*. The HPLC analytical equipment and methods have been previously described *(17)*.

Preparation of purified carotenoid doses

General precautions for work with carotenoids were taken to prevent degradation and artifact formation during the extraction and analytical process *(18)*. PE doses were prepared from PE-rich oil (Vitan Ltd., Dnepropetrovsk, Ukraine) by dissolving the PE-rich oil in chloroform and injecting onto a preparatory HPLC system that has been previously described *(19)*. LYC was extracted from Redivivo 10% LYC beadlets (DSM, Heerlen, Netherlands) using a modified protocol (BASF, *personal communication)* by adding 15 μL alcalase (Calbiochem, San Diego, CA) and a few crystals of BHT to 100 mg of beadlets, then 2.5 mL of 2% aqueous ammonium hydroxide solution was added. The mixture was vortexed briefly and then heated in a shaking 80 °C water bath for 2 min. Five mL ethanol and 2 mL hexanes were added to the solution, it was vortexed, and the hexane layer was removed and reserved. The hexanes extraction was repeated at least 5 times until the hexanes layer was no longer red. The hexanes were combined and evaporated using an AS160 Savant Speedvac (Thermo Scientific, Pittsburgh, PA). The concentrated LYC extract was then reconstituted in chloroform and injected onto the HPLC preparatory system described above. LYC was collected and used for dose preparation. Purified LYC and PE isomeric composition was evaluated and amounts were quantified using the analytical HPLC system described in *Chapter 3*. Purified carotenoids were dispensed into separate vials to be opened fresh on each day of dosing; gerbil dosing was staggered over 9 days at the end of the study, to facilitate the pharmacokinetic aspect of the study. Purified LYC doses consisted of 52 ± 2% in the all *all-E* isomer with the remainder in various *Z* isomers, while 100% of the phytoene was identified to be present in the *15-Z* isomer. Carotenoid doses were reconstituted in cottonseed oil daily, using chloroform to solubilize the carotenoid, adding cottonseed oil, and evaporating the chloroform under a stream of argon gas *(10)*. Complete

evaporation of chloroform was confirmed gravimetrically. Doses were administered to gerbils by oral intubation and the dose mass administered was recorded. Carotenoid dose samples from each day were reserved for confirmatory HPLC analysis.

Serum and tissue HPLC analyses

Serum and tissue extraction methods were previously described *(10)*.

Statistical analyses

Tissue carotenoid concentrations from TPPE and TPLY animals at 6, 12, and 24 hours postdosing were compared to each other and to the TPCO group. Significant differences were determined by using the post-hoc Tukey's studentized range test ($\alpha = 0.05$) within ANOVA when the assumptions of ANOVA were met, otherwise the Wilcoxon and Kruskal-Wallis non-parametric tests were used to evaluate group differences ($\alpha = 0.05$) using the statistical analysis software SAS v. 7.1 (SAS Institute Inc., Cary, NC). All results are presented at an average of the analyses ± the standard error of the mean (SEM), unless otherwise noted.

Results

Animal weight gain

Gerbil final weight did not differ by diet, dosing group, or sacrifice time point ($p = 0.06$). Average gerbil weight at time of sacrifice was 67.8 ± 0.5 g.

Tomato carotenoid biodistribution in tomato powder-fed, cottonseed oil-dosed gerbils

Gerbils from the TPCO group were sacrificed six hours after dosing to evaluate tomato carotenoid bioaccumulation. Prior to dosing, gerbils were fed the control diet for two days to minimize variability of serum and tissue carotenoid concentrations as a result of tomato-powder feeding. The relative proportions of measured tomato carotenoid in the tomato-powder diet were 67% *all-E* LYC, 26% *Z*-LYC isomers, 3% PE, 2% ZC, 1% PF, and 1% BC (Figure 4.2),

however the relative proportions accumulated in tissues differed substantially from that in the tomato powder. The primary carotenoids present in the serum were PE, PF and LYC, with the majority of LYC present as *Z* isomers (58%), and there were lesser amounts of ZC, and BC (Table 4.2, Figure 4.3). Of all tissues analyzed, carotenoids were most concentrated in the liver tissue (Figure 4.4) where LYC was the predominant accumulated carotenoid (74% *Z* isomers, 26% *all-E*) followed by PF, then lesser amounts of PE, ZC, and BC (Table 4.3, Figure 4.5). The most prominent carotenoid in the adrenals was PF, followed by PE, then LYC (83% *Z* and 17% *all-E*), ZC, and BC (Table 4.4, Figure 4.5). Spleen tissue primarily accumulated LYC (59% *Z* and 41% *all-E*), followed by PF, with lower amounts of PE and ZC, and BC was not detected (Table 4.5, Figure 4.5). Both PF and LYC (67% *Z* and 33% *all-E*) were the most prominent carotenoids in the lung tissue followed by ZC then PE, and BC was not detectable (Table 4.6, Figure 4.5). PE was the greatest accumulated carotenoid in the gonadal adipose followed by LYC (57% *Z* and 43% *all-E*), then ZC and PF (Table 4.7, Figure 4.5). Two androgen-sensitive tissues were analyzed and of those two, carotenoid concentrations were about 3.5-times greater in the testes than in the prostate-seminal vesicle complex. In the testes, LYC was most prominent (74% *Z* isomers and 26% *all-E*) followed by PF and ZC, while BC and PE were not detected (Table 4.8, Figure 4.6). Similarly, the prostate and seminal vesicle complex accumulated more LYC (58% *Z* isomers, and 42% *all-E* isomers) than PF and ZC, and BC and PE were not detectable (Table 4.9, Figure 4.6). Tissues and serum of CCO animals were analyzed and carotenoids were not detected in the adrenals, prostate-seminal vesicle complex, serum, or testes, while PE was detected in the liver (1.27 ± 0.37 μg/g) and traces of other carotenoids (3.5 μg total carotenoids/g) were also present, likely due to transient tomato powder

exposure during weighing and cage changes. CCO group also had trace LYC detected in the spleen (0.07 μg/g), and LYC and ZC in the lung tissue (21.43 ng/g and 2.95 ng/g, respectively).

Effect of PE or LYC-dosing on serum and tissue carotenoid concentrations

Serum and tissue carotenoid concentrations were examined at 6, 12, and 24 hours postdosing in TPPE and TPLY groups, and these carotenoid concentrations were compared to the TPCO group sacrificed 6 hours after dosing. Serum PE increased in response to PE dosing and that increase was sustained over the 24 hour period monitored (Table 4.2, Figure 4.7). Serum *Z*-LYC isomers increased in response to LYC dosing and remained greater than the TPCO group for the 24 hour duration while the *all-E* LYC serum concentration was only elevated through 12 hour post-LYC dosing and returned to the TPCO levels at 24 hour. Serum ZC, PF, and ZC were not impacted by carotenoid dosing.

Liver PE concentrations increased in response to PE dosing over the 24 hour time-course, however, neither *Z* nor *all-E* LYC concentrations were impacted by LYC dosing (Table 4.3, Figure 4.7). Liver PF, ZC, and BC concentrations were not affected by carotenoid dosing. Alternatively, adrenal PE was not different in TPPE animals compared to TPCO group, but TPLY animals had greater *Z*-LYC isomer concentrations 12 hours after dosing compared to TPCO animals while *all-E* LYC concentrations did not differ over 24 hours (Table 4.4, Figure 4.7). At 12 hours post-dosing, PE-dosed gerbils had greater PF and ZC adrenal gland concentrations than LYC dosed animals and greater BC than the TPCO animals (Table 4.4). PE dosing led to an increase in spleen PE concentrations over the 24 hr period, but LYC dosing did not increase LYC concentrations in the spleen (Table 4.5). Lung PE concentrations increased 6-fold at 24 hours post-PE dosing, but pulmonary LYC did not increase in response to LYC-dosing. BC, ZC, and PF concentrations in the lung tissue were not affected by carotenoid dosing

(Table 4.6). Carotenoid dosing did not have a significant impact on gonadal adipose carotenoid concentrations over time (Table 4.7). The testes and prostate-seminal vesicle complex did not have detectable PE over the 24 hour time-course (Tables 8 & 9). PE-dosed animals had lower testes *Z*-LYC concentrations 6 and 12 hours post-dosing as well as decreased PF at 6 hours and ZC at 12 hours post-dosing. At 12 hours post PE-dosing, there was greater *all-E* LYC and ZC compared to the TPCO animals.

Discussion

TPCO animals accumulated tomato carotenoids in all of the evaluated tissues as well as in serum. In this study, the gerbils were fed a control diet for two days prior to dosing to allow carotenoids to be cleared from the gastrointestinal tract so that carotenoid interactions were not a factor during the oral dosing study. Although the gerbils had not consumed carotenoids for at least two days before serum was collected, PE, PF, LYC, ZC, and BC were all detected, however they were present at 10-fold or lower concentrations (0.026, 0.022, 0.040, 0.011, and 0.008 ng/μL, respectively) than those previously reported for humans that consumed a high LYC tomato juice for 4 wk (PE, 0.16; PF, 0.39; LYC, 0.40; ZC, 0.14; BC 0.22 ng/μL plasma) *(20)*. Gerbil hepatic carotenoid concentrations (LYC, 72; PF, 36; PE, 13; ZC, 6 μg/g) (Table 4.3) were much higher than those reported in humans (LYC, 352; PF, 261; PE, 168; ZC, 150 ng/g), though the human tissue examined was taken from autopsy specimens and not from a feeding study *(9)*. It may be reasonable to expect that with regular tomato consumption, human liver carotenoid concentrations would be greater than autopsy samples. However, the accumulated hepatic carotenoid profile in gerbils is similar to humans, such that LYC > PF > PE, but with the exception that gerbils accumulated more ZC than BC in contrast to what is observed in humans *(9)*. The adrenal glands had the next greatest carotenoid concentrations after the liver (Table 4.4).

The adrenal glands are a site of steroid synthesis, and thus necessitate high cholesterol uptake via the SR-B1 transporter, which is also known to transport carotenoids *(21)*. Human adrenal glandular carotenoid concentrations have not been previously reported to our knowledge but the findings for gerbils are similar to what is seen in the rat such that PE and PF are nearly equivalent while there are lesser amounts of accumulated LYC and ZC *(10)*. Gerbil splenic carotenoid accumulation (Table 4.5) was similar in the accumulation profile to rats such that LYC was most prominent followed by PF and lesser amounts of ZC and PE, but gerbils accumulated less total carotenoids than the rat *(10)*. Pulmonary carotenoid accumulation in the gerbil (Table 4.6) differed from what has been reported for the human autopsy samples *(9)*. Gerbil pulmonary carotenoid concentrations of LYC and PF were greatest, followed by ZC and PE, while BC was below detection. In humans PE is the major pulmonary tomato carotenoid observed followed by LYC, BC, PF, and ZC *(9)*. Adipose tissue is viewed as a major depository for carotenoids because of the large size of this tissue although there are typically low concentrations of carotenoids *(21)*. Carotenoid concentrations measured in the gonadal adipose tissue site showed that although gerbils accumulated ng/g amounts of tomato carotenoids, the carotenoid profile differed substantially from that observed in rats (gerbils, PE > LYC > ZC > PF; vs. rats, LYC > PF > PE > ZC)*(10)*.

Androgen-sensitive tissues have been previously reported to differ in carotenoid accumulation profiles from other tissues, such that LYC is preferentially accumulated *(9, 10)*. In this current study, the intact prostate-seminal vesicle complex and the testes from gerbils were analyzed for carotenoid content. In human autopsied prostate samples, LYC was most prominent (374 ng/g) followed by PF, ZC, BC, and PE (201, 187, 163, and 45 ng/g, respectively) *(9)*. Similar to humans, LYC is the most prominent carotenoid in the gerbil prostate-seminal

vesicle complex, followed by ZC then PF; BC and PE were not detected. Rats had a different distribution of carotenoids in the seminal vesicles with the major carotenoid being LYC (53.7 ng/g) followed by equal amounts of PE and PF and a lesser amount of ZC *(10)*. Similarly to gerbil prostatic-seminal vesicular carotenoid profile, LYC is the major carotenoid present in gerbil testes followed by PF then ZC. In the rat, LYC and PF are the major testicular carotenoids followed by PE and ZC *(10)*. It appears that in both rats and gerbils, LYC and PF are prominently accumulated in the testes, and LYC is predominant in the prostate. Data on human testicular PE and PF concentrations are not currently available. It is interesting to note the contrast between the steroidogenic tissues examined in that the adrenal gland preferentially accumulates PF and PE, while the androgenic testes accumulate primarily LYC.

Serum and adipose tissue carotenoid concentrations are popular epidemiologic and clinical markers for carotenoid exposure, however our findings along with those for the rat indicate that while serum and adipose are useful to provide indication of *which* carotenoids have been consumed, these measures are not representative of the *amounts* of carotenoids that are accumulated in other tissues. Often, *in vitro* studies base carotenoid dosing levels on serum concentrations when it may, in fact, be more relevant to base dosing levels on tissue concentrations determined in an appropriate animal model.

Oral carotenoid dosing in oil led to concomitant increases of the dosed carotenoid in the serum. PE dosing increased serum PE by 570% at 6 hours, 340% at 12 hours, and 650% at 24 hours, while LYC dosing increased *Z* LYC isomers in serum 90% at 6 hours, 60% at 12 hours, and 30% at 24 hours, while *all-E* LYC was only increased at the 6 hour time-point by 40% and was not significantly different from TPCO animals at the subsequent time-points. In short, the PE dose was absorbed to a greater extent than the LYC dose although the PE dose mass was less

than that of the LYC dose. This difference may be due to PE being present primarily in a *Z*-isomeric conformation. Previous studies with LYC have shown *Z*-isomers to be less prone to aggregation and crystallization and therefore more likely to be incorporated into bile acid micelles *(21)*. While PE-dosing increased serum PE and LYC-dosing increased serum LYC, each for the 24 hour duration, not all tissues showed the same increase in carotenoids in response to dosing. Hepatic and splenic PE increased after PE-dosing in agreement with what was observed in rats provided with a PE-dose *(10)*, however LYC did not increase in response to LYC-dosing in either tissue. The adrenal glands, alternatively, showed an increase in *Z*-LYC isomer accumulation at 12 hours post-PE-dosing, but there was not a rise in adrenal PE during the 24 hour time-course, in agreement with previous findings in rats dosed with PE *(10)*. Pulmonary PE concentrations were elevated 24 hours post-PE-dosing suggesting that this tissue may be subject to a lag in carotenoid uptake after dosing. Carotenoid dosing did not impact gonadal adipose tissue carotenoid concentrations though there was a trend for an increase in adipose PE at the 24 hour timepoint. Taken together, the results suggest that although both LYC and PE were absorbed from the doses, PE was more readily absorbed and accumulated in the liver and spleen, while LYC was accumulated more readily in the adrenal gland tissue. The lung and gonadal adipose tissues were not as responsive to the carotenoid doses, though by 24 hours there was a modest increase in tissue PE in each tissue. These differences in tissue responses to carotenoids may be due to differences in carotenoid packaging into chylomicrons as well as lipoproteins, or absorption into cells. Since the increase in serum LYC was less than that of PE, it suggests that PE may be more easily taken up into intestinal cells or more easily absorbed into the lymphatic blood system than LYC.

PE and LYC concentrations in the androgen-sensitive tissues did not increase significantly in response to the single oral dose. However, when dosed with PE, gerbils had lower testicular *Z*-LYC concentrations compared to the TPCO group, suggesting that the LYC dose may have contributed to maintaining the testicular LYC levels over time. Testicular LYC may have decreased over the 24 hour time span in TPPE animals due to LYC metabolism. Similarly to the testes, prostatic and seminal vesicular PE and LYC were not impacted by corresponding doses. Interestingly, PE was not clearly detected in either of these androgen sensitive tissues, which suggests these tissues may differ in absorption or metabolism of PE, compared to the other tissues examined. The testicular LYC concentrations reported here are much greater that those previously reported for gerbils consuming LYC daily, but the previous study provided a lower levels of LYC (60 μg/d) in the forms of cottonseed oil doses of tomato paste extract, red carrot extract, or purified *all-E* LYC *(14)*, whereas, the gerbils in the present study consumed an estimated 7.5 g of 10% tomato powder diet/d, and thus were receiving approximately 2.23 mg LYC/d.

One of the most striking outcomes of this study is the differential absorption and bioavailability of the different carotenoids from tomato powder. LYC is present in the tomato powder diet at levels 54 times that of ZC, 26 times the level of PE, and 80 times that of PF but does not accumulate in the tissues at such disparate levels, rather LYC is accumulated at similar levels in tissues to these more minor dietary carotenoids. This disconnect between dietary LYC levels and tissue levels may be due to low bioavailability of LYC because of the prominent *all-E* conformation in tomato powder, which is less bioavailable than *Z* isomers; whereas the other carotenoids are found in primarily *Z* isomers *(22)*. The difference may also be due to elevated LYC metabolism. Some evidence from our laboratory suggests that LYC is a substrate for the

CMO II enzyme, while PE and PF are not *(23)*. This was shown when CMOII knockout mice were fed LYC-containing diets and LYC significantly increased in tissues compared to WT and CMOI knockout mice. It should also be noted that the gerbils in this study do not have sterile gastrointestinal tracts, therefore it is possible that gut microbiota played a role in tomato carotenoid bioavailability or metabolism. Gut flora may be important for release of carotenoids from a fibrous matrix *(24, 25)*, but there is little support in the literature for microbial metabolism of carotenoids in the gastrointestinal tract *(26)*. Further research using labeled carotenoid tracers should confirm if there is differential metabolism of PE, PF, and LYC in gerbil tissues.

In conclusion, we have shown that gerbils fed a 10% tomato powder diet for 26 days accumulated biologically relevant tissue concentrations of tomato carotenoids when compared with concentrations reported in humans. Additionally, we have shown that the dietary carotenoid profile did not directly relate to gerbil tissue carotenoid profiles. A possible explanation for differences in bioaccumulation of these linear carotenoids may be due to differential bioavailability of carotenoids as demonstrated by the PE and LYC dosing experiment, which showed PE is more bioavailable than LYC when delivered in an oil dose. Alternatively, LYC may be a substrate for the CMO II enzyme, which may explain lower LYC concentrations observed in gerbils fed high amounts of LYC. Further studies utilizing radiolabeled PE and LYC will answer several remaining questions. Radioactively labeled carotenoids offer the advantage of being able to be identified at low tissue concentrations and in altered metabolic product forms. For example, by providing ^{14}C-PE, it will be determined if PE is being taken up into the testes and being metabolized or not. Additionally, labeled LYC dosing

will indicate if LYC is more readily metabolized in the gerbil than PE providing an explanation for lower concentrations of LYC than PE in the gerbil in response to dosing.

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Figures

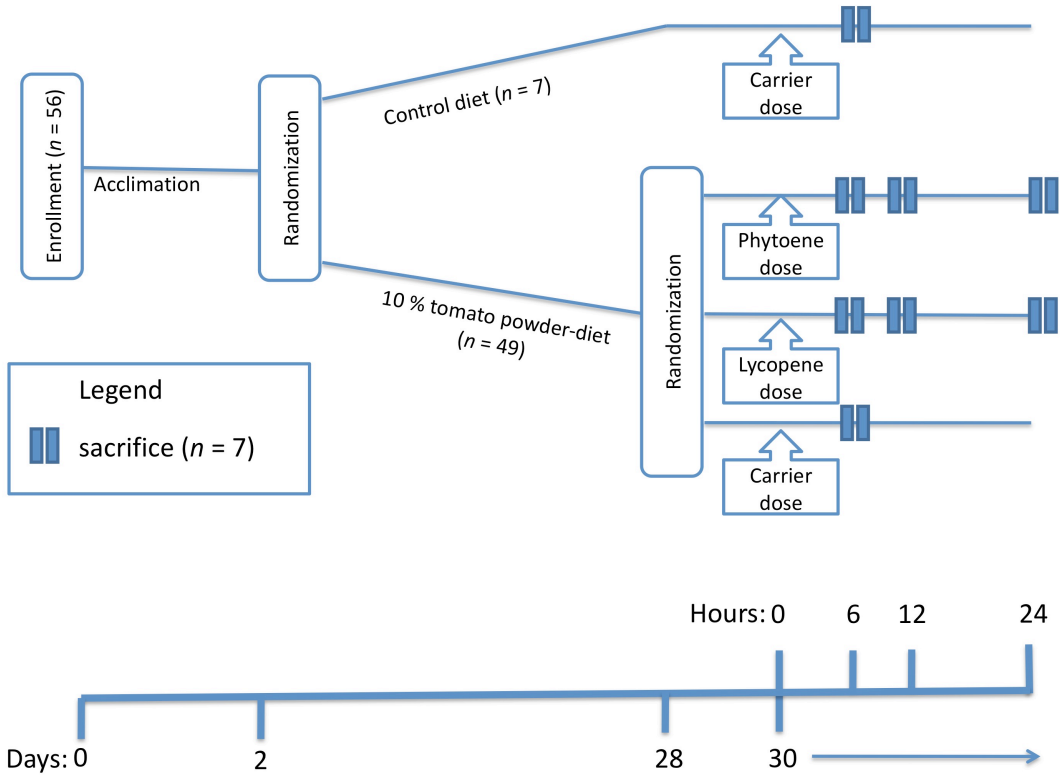

Figure 4.1. General study design for gerbil feeding and carotenoid dosing study. Gerbils (n = 56) were adapted to their new environment and to powdered diet over 2 days. For the subsequent 26 days they were fed either control diet (n = 7) or 10% tomato powder diet (n = 49). After 26 days, all gerbils were fed a control diet for 2 days. On day 30, the gerbils were dosed (gastric intubation) with either cottonseed oil, PE in cottonseed oil, or LYC in cottonseed oil. Animals were sacrificed 6, 12, or 24 hours postdosing.

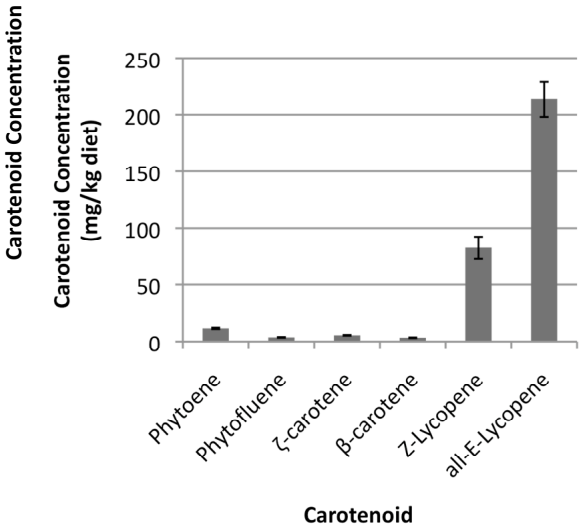

Figure 4.2. Carotenoid concentrations in the 10% tomato powder diet. Bars respresent the average of three replicates and errors bars represent the standard error of the mean.

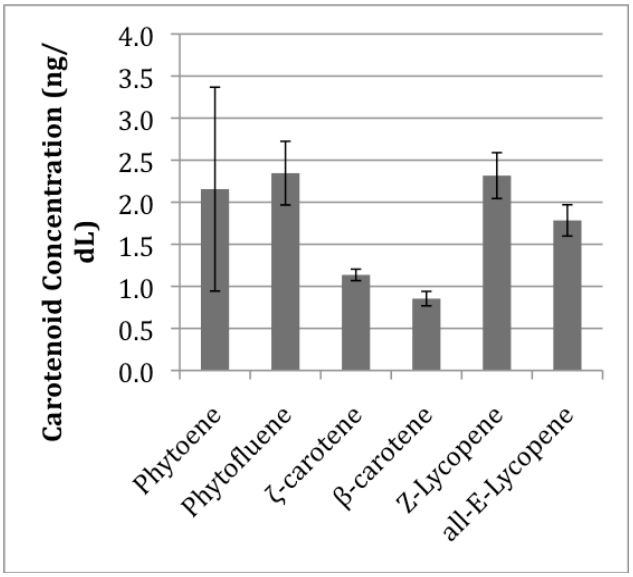

Figure 4.3. Serum carotenoid concentrations in gerbils fed a 10% tomato powder diet for 26 days followed by 2 days of control diet-feeding.

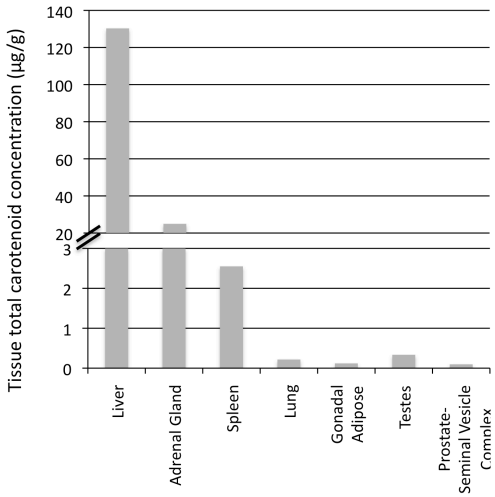

Figure 4.4. Total carotenoid concentrations in various gerbil tissues in response to feeding a 10% tomato powder diet for 26 days followed by 2 days of control diet-feeding.

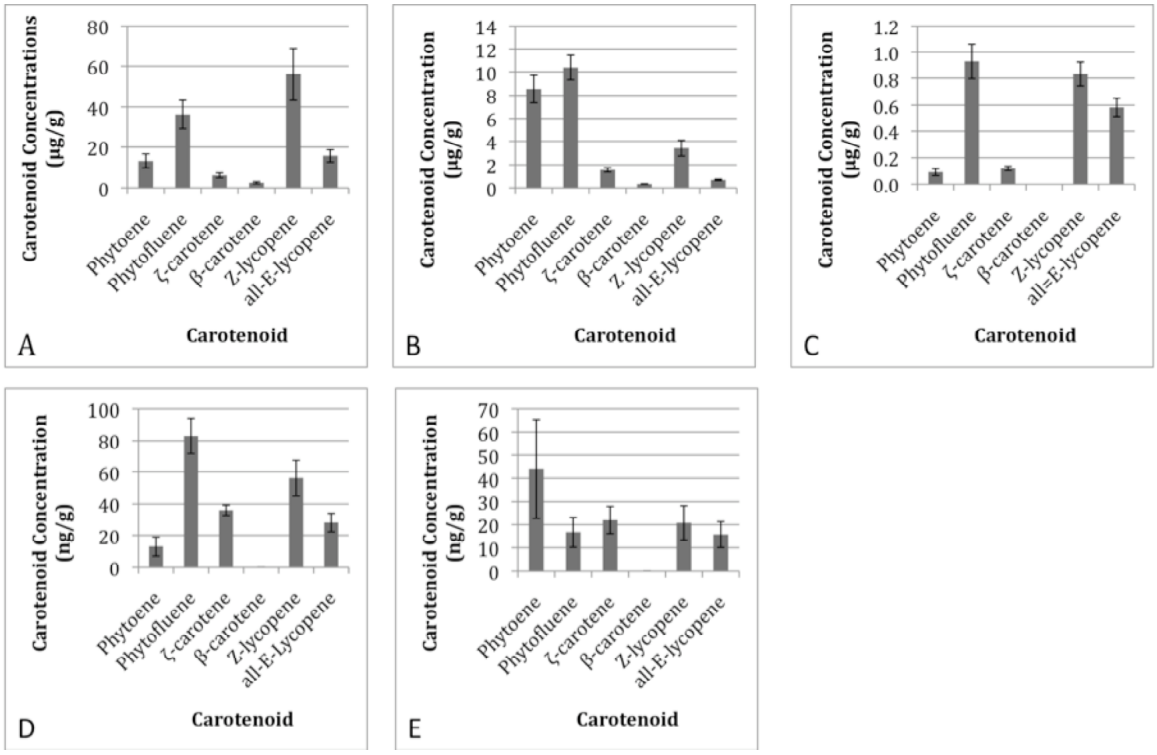

Figure 4.5. Carotenoid accumulation in (A) liver, (B) adrenal glands, (C) spleen, (D) lung, and (E) gonadal adipose in response to tomato powder feeding for 26 days followed by 2 days of control diet consumption. Bars represent the average of seven sample analyses and error bars represent the standard error of the mean.

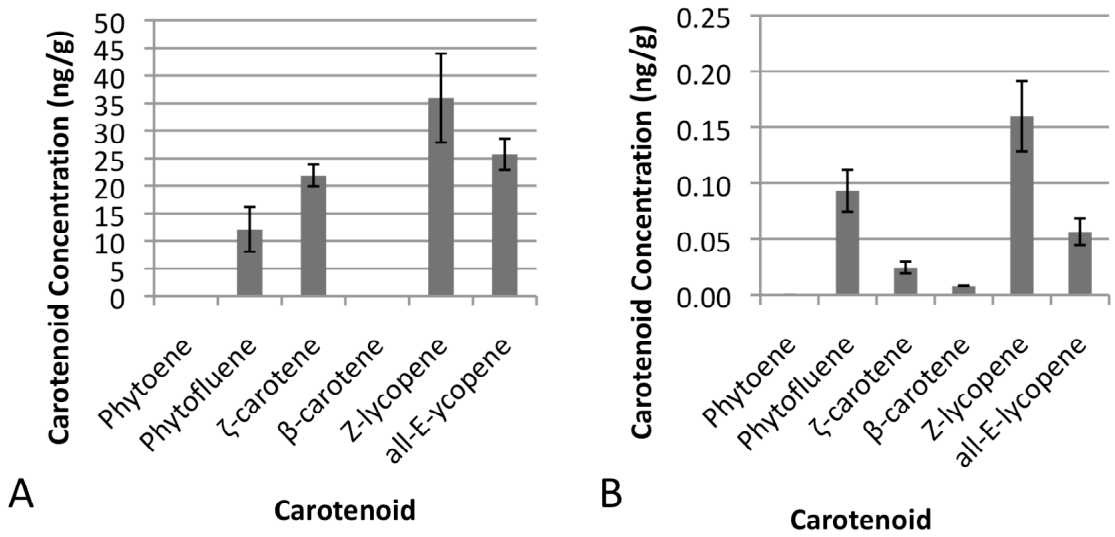

Figure 4.6. Carotenoid accumulation in the (A) prostate-seminal vesicle complex and (B) testes in response to tomato powder feeding for 26 days followed by 2 days of control diet consumption. Bars represent the average of seven sample analyses and error bars represent the standard error of the mean.

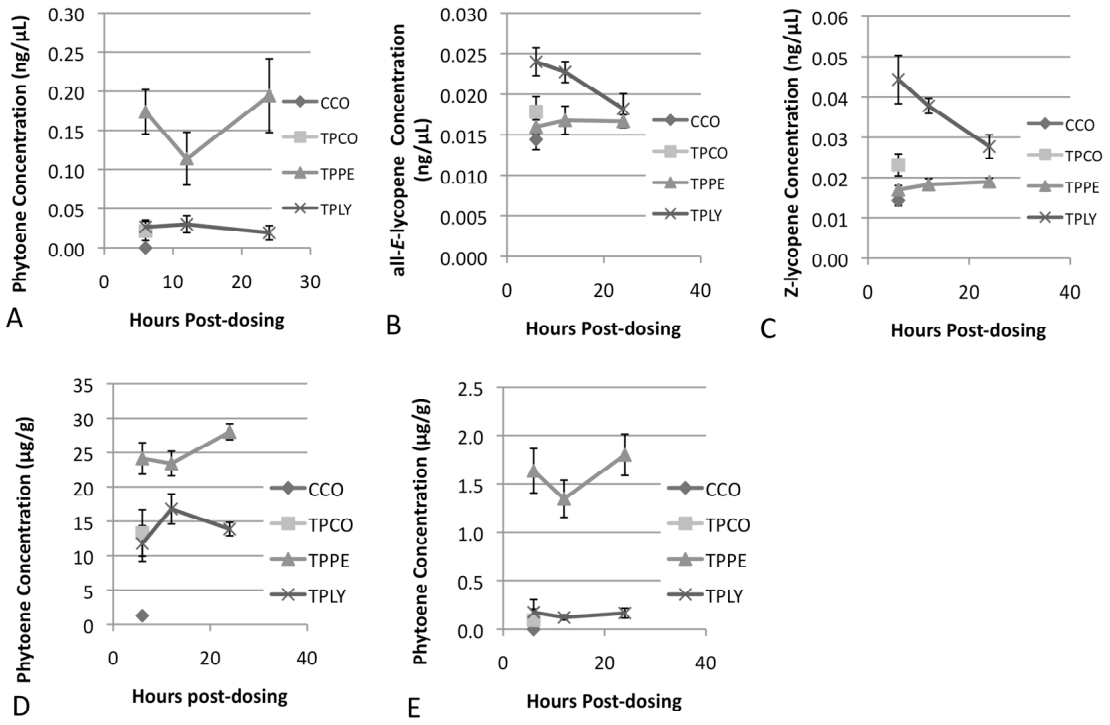

Figure 4.7. Carotenoid concentrations time-course over 24 hours in gerbil serum (A-C), liver (D), and spleen (E) after being pre-fed either a control diet and dosed with cottonseed oil (CCO), or pre-fed with 10% tomato powder diet and dosed with cottonseed oil (TPCO), phytoene in oil (TPPE), or lycopene in oil (TPLY). Each data point represents the average of 7 sample analyses and error bars represent standard error of the mean.

Tables

Table 4.1. Gerbil diet composition.

Component	Control Diet g/kg	10% Tomato Powder Diet g/kg
Sucrose	361	319.78
Casein [1]	200	186.1
Cornstarch	150	150
Maltodextrin	120	108.96
Cottonseed Oil	60	55.14
Cellulose	60	43.81
Mineral Mix [2]	35	35
Vitamin Mix [3]	5	5
L-cystine	3	3
Choline bitartrate	2.5	2.5
CaHPO4	2	2
MgO	1.75	1.75
Tomato Powder [4]	0	100
kcal/g	3.78	3.78

[1]Vitamin-free test casein, contains 0.9 g protein/g casein.

[2]AIN-93G-MX (Teklad) provides calcium carbonate (12.495 g/kg diet); potassium phosphate, monobasic (6.86 g/kg diet); potassium citrate, monohydrate (2.48 g/kg diet); sodium chloride (2.59 g/kg diet); potassium sulfate (1.63 g/kg diet); magnesium oxide (0.85 g/kg diet); ferric citrate (0.21 g/kg diet); zinc carbonate (0.06 g/kg diet); manganous carbonate (0.02 g/kg diet); cupric carbonate (0.01 g/kg diet); potassium iodate (3.5 *10^-4 g/kg diet); sodium selenate (3.605*10^-4 g/kg diet); ammonium paramolybdate, tetrahydrate (2.8*10^-4 g/kg diet); sodium meta-silicate, nonahydrate (0.0575 g/kg diet); chromium potassium sulfate, doceahydrate (9.6*10^-3 g/kg diet); lithium chloride (6.09 * 10^-4 g/kg diet); boric acid (2.85*10^-3 g/kg diet); sodium fluoride (2.22*10^-3 g/kg diet); nickel carbonate hydroxide, tetrahydrate (1.11 *10^-4 g/kg diet); ammonium meta-vanadate (2.31*10^-4 g/kg diet); and sucrose (7.73 g/kg diet) *(27)*.

[3]AIN-93-VX (Teklad) provides niacin (0.015 g/kg diet); calcium pantothenate (0.008 g/kg diet); pyridoxine HCl (3.5 *10^-3 g/kg diet); thiamin HCl (3*10^-3 g/kg diet); riboflavin (3*10^-3 g/kg diet); folic acid (1*10^-3 g/kg diet); biotin (1*10^-4 g/kg diet); vitamin B_{12} (0.1% in mannitol) (0.0125 g/kg diet); vitamin E, DL-alpha tocopheryl

Table 4.1. continued.

acetate (500 IU/g) (0.075 g/kg diet); vitamin A palmitate (500,000 IU/g) (0.004 g/kg diet); vitamin D_3 cholecalciferol (500,000 IU/g) (0.001 g/kg diet); vitamin K_1, phylloquinone (3.75 *10^-4 g/kg diet); sucrose (4.87 g/kg diet) *(27)*.

[4]Drum dried tomato powder (Futureceuticals, Momence, IL) contains approximately 3.68 kcal/g, 0.01 g protein/g, 0.03 g fat/g, 0.16 g fiber/g, and 0.52 g carbohydrate/g.

Table 4.2. Gerbil serum carotenoid concentrations [1, 2].

Hours Post-dosing:		***6***		***12***		***24***	
mean ± SEM (ng/dL)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene $	2.6 ± 1.2	2.8 ± 0.8 A	17.4 ± 2.8 *, B	2.5 ± 1.1 A	11.4 ±3.4 *, B	2.2 ± 1.0 A	19.4 ± 4.7 *, B
Z-Lycopene isomers $	2.3 ± 0.3	4.3 ± 0.7 *, B	1.7 ± 0.1A	3.7 ± 0.2 *, B	1.8 ± 0.1A	3.0 ± 0.3*	1.9 ± 0.1
all-E Lycopene	1.7 ± 0.2	2.3 ± 0.2 *, B	1.6 ±0.1	2.3 ± 0.1B	1.7 ± 0.2A	2.1 ± 0.3	1.7 ± 0.1
Phytofluene	2.2 ± 0.3	2.6 ± 0.6	2.2 ± 0.3	2.2 ± 0.2	1.8 ± 0.2	3.3 ± 0.6	2.5 ± 0.3
Zeta-carotene	1.1 ± 0.1	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.4 ± 0.2	1.3 ± 0.1
Beta-carotene	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1

[1] *n* = 6-7/dosing group; carotenoids were provided in 1 mg doses.[2] Significant differences between PE and LYC animals at different time points versus cotton-seed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.
($ except for differences in phytoene and *cis*-lycopene concentrations where the Kruskal-Wallis non-parametric t-test due to a violation of homogeneity of variance)

Table 4.3. Gerbil liver carotenoid concentrations [1, 2]

Hours Post-dosing:		***6***		***12***		***24***	
mean ± SEM (μg/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	13.31 ± 3.38	11.77 ± 2.59A	24.14 ± 2.29*,B	16.80 ± 2.18	22.03 ± 2.14	13.87 ± 0.97A, $	27.98 ± 1.12*,B, $
Z-Lycopene isomers	56.11 ± 12.75	49.69 ± 9.72	63.65 ± 6.18	66.55 ± 7.61	50.76 ± 0.86	56.46 ± 5.20	45.74 ± 6.63
all-E Lycopene	15.76 ± 2.98	13.28 ± 1.96	17.67 ± 0.96	19.53 ± 2.98	16.65 ± 2.42	15.12 ± 1.21	14.74 ± 1.70
Phytofluene	36.28 ± 7.11	28.36 ± 4.39	42.06 ± 3.15	38.67± 4.60	32.15 ± 2.92	38.85 ± 2.51	32.11 ± 2.30
Zeta-carotene	6.26 ± 1.20	4.81 ± 0.82	6.68 ± 0.84	6.25 ± 0.71	5.35 ± 0.38	6.55 ± 0.40	5.99 ± 1.04
Beta-carotene	2.58 ± 0.46	2.37 ± 0.40	2.67 ± 0.29	2.38 ± 0.26	2.04 ± 0.08	2.76 ± 0.14	2.20 ± 0.12

[1] *n* = 6-7/dosing group

[2] Significant differences between PE and LYC animals at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.

Table 4.4. Gerbil adrenal carotenoid concentrations[1, 2]

Hours Post-dosing:		**6**		**12**		**24**	
mean ± SEM (μg/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	8.54 ± 1.16	7.17 ± 0.73	9.41 ± 0.92	6.58 ± 0.64	12.78 ± 0.92	9.12 ± 1.02	11.49 ± 1.18
Z-Lycopene isomers	3.41 ± 0.67	3.40 ± 0.60	3.86 ± 1.12	2.35 ± 0.46A	3.95 ± 0.54*,B	3.07 ± 0.56	2.65 ± 0.53
all-E Lycopene	0.71 ± 0.08	0.62 ± 0.07	0.68 ± 0.14	0.62 ± 0.06	0.69 ± 0.05	0.71 ± 0.13	0.67 ± 0.10
Phytofluene	10.38 ± 1.08	10.13 ± 1.06	10.34 ± 1.00	8.14 ± 0.59A	12.02 ± 0.87B	10.95 ± 1.12	7.13 ± 1.33
Zeta-carotene	1.57 ± 0.19	1.60 ± 0.22	1.67 ± 0.18	1.23 ± 0.12A	1.80 ± 0.13B	1.75 ± 0.22	2.60 ± 0.13
Beta-carotene	0.34 ± 0.02	0.33 ± 0.03	0.40 ± 0.05	0.40 ± 0.02	0.44 ± 0.03*	0.40 ± 0.04	0.36 ± 0.04

[1] *n* = 6-7/dosing group

[2] Significant differences between PE and LYC-dosed animal tissue concentrations at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.

Table 4.5. Gerbil spleen carotenoid concentrations[1, 2]

Hours Post-dosing:		**6**			**12**		**24**
mean ± SEM(μg/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	0.09 ± 0.03	0.17 ± 0.03A	1.64 ± 0.23*B	0.12 ± 0.03A	1.35 ± 0.19*B	0.17 ± 0.06A	1.56 ± 0.30*B
Z-Lycopene isomers	0.83 ± 0.09	1.19 ± 0.21	1.02 ± 0.29	1.28 ± 0.13	0.98 ± 0.15	1.21 ± 0.19	1.42 ± 0.25
all-E Lycopene	0.58 ± 0.07	0.80 ± 0.17	0.71 ± 0.17	0.97 ± 0.22	0.71 ± 0.06	0.75 ± 0.11	0.92 ± 0.10
Phytofluene	0.93 ± 0.13	1.28 ± 0.20	1.12 ± 0.22	1.23 ± 0.10	1.14 ± 0.16	1.32 ± 0.05	1.59 ± 0.17*
Zeta-carotene	0.12 ± 0.02	0.17 ± 0.03	0.13 ± 0.03	0.14 ± 0.01	0.14 ± 0.02	0.20 ± 0.01	0.23 ± 0.03
Beta-carotene	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[1] n = 6-7/dosing group

[2] Significant differences between PE and LYC-dosed animal tissue concentrations at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.

Table 4.6. Gerbil Lung Carotenoid Concentrations [1, 2]

Hours Post-dosing:		**6**		**12**		**24**	
mean ± SEM (ng/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene $	12.97 ± 5.86	36.21 ± 26.58	15.86 ± 4.58	9.32 ± 5.40	25.63 ± 16.78	20.32 ± 5.10A	91.04 ± 36.07B,*
Z-Lycopene isomers	56.26 ± 11.18	52.52 ± 5.56	48.11 ± 4.54	60.67 ± 7.69	38.72 ± 3.58	59.77 ± 13.44	62.64 ± 17.65
all-E Lycopene	28.11 ± 5.89	31.98 ± 1.44	28.02 ± 1.84	32.90 ± 1.89	25.40 ± 2.50	35.10 ± 4.82	35.29 ± 5.70
Phytofluene	82.96 ± 11.28	73.24 ± 9.92	63.54 ± 7.01	60.99 ± 6.52	48.67 ± 4.14	80.88 ± 6.00	83.79 ± 18.21
Zeta-carotene	36.03 ± 3.22	38.97 ± 2.05	35.66 ± 2.26	32.90 ± 0.88	29.75 ± 2.16	38.97 ± 1.92	39.49 ± 4.37
Beta-carotene	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[1] *n* = 6-7/dosing group

[2] Significant differences between PE and LYC-dosed animal tissue concentrations at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.

$ except for differences in phytoene concentrations where the Kruskal-Wallis non-parametric t-test due to a violation of homogeneity of variance

Table 4.7. Gerbil Gonadal Adipose Carotenoid Concentrations[1, 2]

Hours Post-dosing:		**6**			**12**		**24**
mean ± SEM(ng/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	44.12 ± 21.39	58.19 ± 21.01	55.77 ± 21.09	64.06 ± 21.86	46.86 ± 18.62	40.59 ± 9.87	86.78 ± 30.24
Z-Lycopene	20.75 ± 7.45	19.44 ± 9.78	10.12 ± 6.57	22.16 ± 5.80	18.01 ± 8.72	14.01 ± 8.87	25.33 ± 8.31
all-E Lycopene	15.69 ± 5.62	12.05 ± 5.76	7.21 ± 4.68	14.13 ± 5.03	11.80 ± 5.58	8.56 ± 5.41	18.30 ± 5.92
Phytofluene	16.69 ± 6.48	15.10 ± 5.38	18.40 ± 4.40	9.49 ± 5.23	11.10 ± 5.71	17.92 ± 11.38	18.35 ± 6.52
Zeta-carotene	21.98 ± 5.94	31.23 ± 2.74	29.16 ± 1.69	26.07 ± 2.48	17.95 ± 6.48	25.28 ± 6.04	26.34 ± 5.61
Beta-carotene	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[1] *n* = 6-7/dosing group; carotenoids were provided in ~1 mg doses.
[2] Significant differences were not detected.
n/d = not detected

Table 4.8. Gerbil Testes Carotenoid Concentrations[1, 2]

Hours Post-dosing:	**6**			**12**		**24**	
mean ± SEM(ng/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Z-Lycopene	159.86 ± 31.52	84.86 ± 20.47	65.16 ± 21.98*	116.80 ± 26.14	56.81 ± 17.68*	84.22 ± 22.37	76.89 ± 20.70
all-E Lycopene	56.52 ± 11.73	33.76 ± 6.35	29.93 ± 8.24	42.59 ± 9.02	27.83 ± 6.39	33.21 ± 7.46	36.87 ± 6.87
Phytofluene	93.28 ± 18.63	64.38± 10.09	43.20 ± 7.61*	64.22 ± 9.93	48.60 ± 9.44	93.28 ± 18.63	76.18 ± 10.74
Zeta-carotene	24.61 ± 5.36	16.92 ± 5.18	12.09 ± 4.56	18.49 ± 4.12	8.94± 3.10*	17.51 ± 3.88	15.47 ± 3.04
Beta-carotene	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[1] *n* = 6-7/dosing group

[2] Significant differences between PE and LYC-dosed animal tissue concentrations at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). No significant differences between PE and LYC dosed groups at a specific time points were detected Significant differences were detected using Tukey's Studentized Range test within ANOVA.

n/d = not detected

Table 4.9. Gerbil Prostate-Seminal Vesicle Complex Carotenoids [1, 2]

Hours Post-dosing:		**6**		**12**		**24**	
mean ± SEM(ng/g)	*Cottonseed Oil*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*	*Lycopene Dose*	*Phytoene Dose*
Phytoene	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Z-Lycopene	35.94 ± 8.07	35.57 ± 5.31	30.46 ± 3.18	24.48 ± 2.18	39.52 ± 4.20	41.77 ± 13.28	31.06 ± 4.54
all-E Lycopene	25.74 ± 2.83	26.42 ± 3.10	26.29 ± 2.42	19.78 ± 1.58A	35.62 ± 4.96B	29.79 ± 5.07	26.30 ± 3.58
Phytofluene	12.12 ± 4.07	13.43 ± 5.23	9.54 ± 2.43	8.60 ± 1.34	8.22 ± 2.09	21.39 ± 9.47	9.41 ± 3.97
Zeta-carotene	21.88 ± 1.97	24.26 ± 3.34	23.64 ± 1.80	17.33 ± 1.48A	30.80 ± 4.12B	27.04 ± 3.68	23.77 ± 3.14
Beta-carotene	n/d	n/d	n/d	n/d	n/d	n/d	n/d

[1] *n* = 6-7/dosing group

[2] Significant differences between PE and LYC-dosed animal tissue concentrations at different time points versus cottonseed oil dosed animals at 6 hr post-dosing are denoted by an asterisk (*). Significant differences between PE and LYC dosed groups at a specific time point are denoted by a capital letter. Significant differences were detected using Tukey's Studentized Range test within ANOVA.

n/d = not detected

CHAPTER 5:
Overall impact of the dissertation accomplishments on the scientific field

Although tomatoes are a complex mixture of biochemicals, LYC has been assumed to be the major bioactive phytochemical in this fruit. In order to better understand the contribution tomato carotenoids, including LYC, PE, PF, and ZC, to human health, a system for production of isotopically labeled LYC, PE, and PF tracers for use in mammalian carotenoid metabolism research was developed. This work also sought to determine the bioaccumulation of tomato carotenoids in an appropriate animal model of human carotenoid bioavailability, the Mongolian gerbil.

This dissertation work has successfully established methods for efficient production of ^{14}C- and ^{13}C-biolabeled carotenoids derived from tomato cell cultures treated with bleaching herbicides. For animal and cell culture trials, production of ^{14}C-carotenoids is most appropriate. To most efficiently produce ^{14}C-carotenoids from tomato cell cultures, cultures should be dosed on day 1 of the 14 day growth cycle with ^{14}C-sucrose and should be treated with an herbicide to promote carotenoid accumulation. If labeled LYC is desired, cell cultures should be treated with CPTA on day one. If labeled PE and PF are needed, cultures should be dosed with norflurazon on day 7 of the growth cycle. If labeled LYC, PE, and PF are all needed these carotenoids can be simultaneously produced by treating cultures with CPTA and norflurazon. For human metabolic tracing studies, ^{13}C-labeled carotenoids may be produced using one of the herbicide treatments and cultures can be grown with uniformly labeled ^{13}C-glucose to yield highly isotopically enriched carotenoids. The availability of this tool for carotenoid tracer production will allow future advances in tomato carotenoid absorption and metabolism research, which was previously not possible.

Through feeding gerbils 10% tomato powder diets and analyzing tissue and serum carotenoid accumulation, we have demonstrated that LYC, PE, PF, and ZC are bioavailable from tomato powder and are accumulated in substantial quantities (ng/g-μg/g), although not in the same ratios in each tissue. By dosing gerbils with either PE or LYC, it was determined that over a 24 hr time period PE was more readily absorbed and led to greater tissue accumulation than LYC. These differences in PE and LYC accumulation are hypothesized to be due to differences in bioavailability and/or metabolism.

Future studies should be performed to continue to improve on tomato cell culture methodologies as a source of isotopically-labeled tracers. Such studies should focus on enhanced enrichment of isotopically-labeled carotenoids by sequentially growing tomato cells with [U]-^{13}C-glucose to approach uniformly-labeled carotenoid production. Additionally, a search for alternate approaches to increase yields of PF should be performed. To follow-up on PE and LYC bioaccumulation observations, the gerbil should be provided with ^{14}C-PE and ^{14}C-LYC doses to elucidate how these carotenoids are metabolized. Similarly, based on findings obtained in gerbils, humans should be provided with ^{13}C-PE, LYC, and PF to determine the relative bioavailability and metabolism of these carotenoids. Collectively, this dissertation research contributed tools for elucidating the biochemical mechanisms of tomato carotenoid-mediated prevention and attenuation of serious chronic diseases, including cardiovascular disease and prostate cancer.

AUTHOR'S CURRICULUM VITAE

Education

University of Illinois at Urbana-Champaign, Urbana, IL
Ph. D. in Nutritional Sciences, expected graduation date: September 2010
Dissertation Title: Production and utilization of phytoene, phytofluene, and lycopene tracers for bioavailability, biodistribution, and metabolic research.
Dissertation Adviser: Dr. John W. Erdman, Jr. ***GPA:*** 3.49/4.0
B.S. in Molecular and Cellular Biology with Distinction, Chemistry minor,
May 2005 ***GPA:*** 3.6/4.0

Research Interests

Carotenoid bioavailability / carotenoid chemistry and analysis / metabolic tracing / isotopically labeled tracers / diet and cancer / plant bioactive components

Research Experience

Doctoral student, Laboratory of John W. Erdman, Jr., 2005-2010

- Developed tomato cell suspension culture systems for production of isotopically biolabeled carotenoids.
- Participated in writing funded NIH R21 grant titled, "Novel 13 -C Tomato Carotenoids for Absorption and Metabolism Studies in Humans "
- Researched tomato carotenoid absorption kinetics and metabolism in the Mongolian gerbil.
- Mentored junior graduate students and managed undergraduate research assistants.

Undergraduate student researcher and research assistant, Laboratory of Mary Ann Lila, 2004-2005

- Established red clover callus and cell suspension cultures for isoflavone production.
- Wrote undergraduate research thesis.
- Participated in plant *in vitro* technology and microphysiology lab maintenance and research.

Technical Skills

- HPLC equipment operation-*advanced*
- HPLC data analysis-*advanced*
- Microsoft Office- Word, Excel, Powepoint- *advanced*
- Phytochemical extraction from plants and animal tissues.
- Mass spectrometry data analysis
- Rodent dissection
- Rodent care
- Plant cell culture techniques
- Plant care
- Animal custom diet formulation
- SAS statistical software
- Radioactive material handling

Publications:

Engelmann, N.J., Campbell, J.K., Rogers, R.B., Rupassara, S.I., Garlick, P.J., Lila, M.A., Erdman, J.W., Jr. Screening and selection of high carotenoid producing in vitro tomato cell culture lines for [(13)C]-carotenoid production. *J. Agric. Food Chem.* **2010,** *58,* 9979-9987.

Engelmann, N.J., Rogers, R.B., Lila, M.A.; Erdman, J.W., Jr. Herbicide treatments alter carotenoid profiles for ^{14}C tracer production from tomato (Solanum lycopersicum cv. VFNT cherry) cell cultures. *J. Agric. Food Chem.* 2009**,** *57,* 4614-4619.
Engelmann, N.J., Reppert, A., Yousef, G.G., Rogers, R.B., Lila, M.A. In vitro production of radiolabeled red clover (*Trifolium pratense*) isoflavones. *Plant Cell Tiss Organ Cult* 2009**,** *98,* 147-156.
Lu, C.H., **Engelmann, N.J.**, Lila, M.A., Erdman, J.W., Jr. Optimization of lycopene extraction from tomato cell suspension culture by response surface methodology. *J. Agric. Food Chem.* 2008**,** *56,* 7710-7714.
Campbell, J.K., **Engelmann, N.J.**, Lila, M.A., Erdman, J.W., Jr. Phytoene, phytofluene, and lycopene from tomato powder differentially accumulate in tissues of male Fisher 344 rats. *Nutr. Res.* 2007**,** *27,* 794-801.
Engelmann, N.J. "Identification, Quantification, and Elicitation of Red Clover Isoflavones from Cell Cultures" senior thesis, University of Illinois-Urbana/Champaign 2005

Abstracts:

Engelmann, N.J., Rogers, R.B., Rupassara, S.I., Garlick, P.J., Lila, M.A., Erdman, Jr.,John W., Jr. Production of [C]-lycopene from high lycopene tomato cell suspension cultures. *Submitted to FASEB J.* 2010**,** *in press.*

Engelmann, N.J., Rogers, R.B., Rupassara, S.I., Garlick, P.J., Lila, M.A., Erdman, Jr.,John W., Jr. [13C]-Carotenoid Production from *hp-1* Tomato Cell Cultures for Human Metabolic Tracing. *FASEB J.* 2009**,** *23,* 896.3.

Engelmann, N.J., Rogers, R.B., Lila, M.A., Erdman, J.W.,Jr Production of distinct carotenoid mixtures from tomato cell suspension cultures varies by herbicide treatment and variety used. *2008 Experimental Biology meeting abstracts [on CD-ROM]* **2008**

Lu, C., **Engelmann, N.J.**, Lila, M.A., Erdman, J.J.W. Optimization of Lycopene Extraction from Tomato Cell Culture. *2008 Experimental Biology meeting abstracts [on CD-ROM]* **2008**

Engelmann, N.J., Rogers, R.B., Lila, M.A., Erdman, J.W.,Jr Production of 14C tomato carotenoids from 'VFNT' Cherry cell suspension cultures treated with herbicidal enzyme inhibitors *The FASEB Journal* 2007**,** *21,* A352.

Engelmann, N.J., Campbell, J.K., Rogers, R.B., Lila, M.A., Erdman, J.W. ,Jr Selection, characterization, and herbicidal enzymatic inhibition of high carotenoid producing tomato cell suspension cultures. *The FASEB Journal* 2007**,** *21,* A 352.

Campbell, J.K., **Engelmann, N.J.**, Rogers, R.B., Lila, M.A., Erdman, J.W., Jr Innovative in vitro plant methodologies for biosynthesis and isolation of tomato carotenoids. *FASEB J.* 2006**,** *20,* A1059-b.

Honors

Scholarships:
- Jonathan Baldwin Turner Scholarship 2001-2002, 2002-2003
- Marshall and Ann Tudor Scholarship, 2002-2003, 2003-2004
- Institute of Food Technologists Scholarship 2001-2002
- Willard Corbett Scholarship: 2001-2002

Fellowships:

- James Scholar Graduate Student Fellowship, 2005-2007

Awards:

University of Illinois

- James L. Robinson Nutrition Impact Award, Division of Nutritional Sciences, 2010
- W.C. Rose Endowed Award, Division of Nutritional Sciences, 2009
- Travel Award, Graduate College, 2007, 2009
- Nutritional Sciences Symposium Poster Competition 2007- 2010
- Margin of Excellence Research Award, Division of Nutritional Sciences, 2006-2009
- Margin of Excellence Travel Award, Division of Nutritional Sciences, 2006-2009

Societies

- Student In Vitro Plant Poster Competition, Society for In Vitro Biology, 2008
- Carotenoid Researchers Interactive Group Poster Competition, American Society for Nutrition, 2007, 2009, 2010
- Dietary Bioactives RIS Poster Competition, American Society for Nutrition, 2007

Professional Affiliations and Service:

American Society for Nutrition - Student Member 2006-Present

- **Nutritional Sciences Council, Student Representative, 2009-present**
  - Member of website development committee.
  - Elected to position by Nutrition Sciences Council members.
- **Dietary Bioactive Compounds Student Representative, 2007-2008**
  - Member of poster competition and business meeting planning committee.
- **Diet and Cancer Student Representative, 2008-2009**
  - Member of poster competition and business meeting planning committee.

Society for In Vitro Biology- Student Member 2005-Present

- **Student Committee, Chair, 2007-2008- performed the following roles:**
  - **Session Co-Convener**, Session title "Non-Technical Skills for the Working Scientist"- prepared for 2008 World Congress, Tucson, AZ.
  - **Workshop Co-Convener**, Workshop title "Student Professional Development - CV/Resume Writing and Interviewing Skills"- prepared for 2008 World Congress, Tucson, AZ.

University Service:

Division of Nutritional Sciences

- **Recruiting weekend, 4 years**
  - Acted as recruiter for prospective students including conducting tours and attending prospective student meals.
- **Regularly meet with guest speakers**
  - Met with and learned about many various guest speakers' careers as well as represent our program.
- **DNS concentration formation discussion, 2007**
  - Attended organizational meetings for DNS Diet and Cancer group.
- **Noontime Nutrition**
  - Founding member of qualifying exam study program "Noontime Nutrition."

- Managed 1.5 years.
- Group persists for 5 years.

- **DNS Director Student Lunches**
  - Co-facilitated discussions with DNS director candidates.
  - Compiled student feedback to be included in a letter to the search committee head.

Nutritional Sciences Graduate Student Association:

- **Co-treasurer, 2006-2008**
  - Initiated fundraising program with intent of raising enough funds to sustain following year of non-Symposium NSGSA activities.
  - Chaired fundraising committee for Summer 2007 - Participated in Bake-off and Garage Sale.
  - Other responsibilities:
    - Manage funds for organization through the on-campus bank.
    - Ensure the fiscal responsibility and financial management of the organization in compliance with the Student Code.
    - Ensure the compliance with all University, local, state and federal rules and regulations.
    - Ensure adequate funds are raised and saved for Symposium through SORF funding requests, and UIUC departmental and corporate donations.

- **Committee Involvement**
  - Outstanding Faculty Award, 2009
  - Student Professional Development, 2008-2009
  - Quiz Bowl, 2005, 2006
  - Holiday Party, 2005, 2006
  - Nutritional Sciences Symposium, 2007-2009
  - Nutritional Sciences Picnic, 2005, 2006
  - New Student Welcome, 2006-2008, Developed a summer welcome party for students who start in the summer. Participated in organization of Fall and Spring welcome parties.
  - Fundraising activities, 2007-2008

<u>Teaching Experience</u>

Discussion Leader, Introduction to Human Nutrition- FSHN 220

- Spring 2008, 2009
- Cited on *List of Teachers Ranked as Excellent by Their Students*, 2008, 2009

Guest lecturer, Introduction to Human Nutrition- FSHN 220

- Lectured and wrote corresponding test questions, Spring 2008.

Teaching Assistant, Scientific Basis for DRIs- NUTR 510

- Worked with Professors Fahey, Garlick, and Erdman to provide study questions for students and was available to answer student questions before and after class, Spring 2009.

Center for Teaching Excellence

- Earned Graduate Teaching Certificate from the Graduate Teaching College.

ACES Teaching College Course

- Earned teaching certification.

ExplorACES
- Designed and presented Functional Foods display to teach the public about bioactive food components and disease prevention, 2007, 2008.

<u>Mentoring Experience</u>

Mentoring undergraduate research assistants, *2005-2010*
- Worked with, taught lab skills to, and mentored 4 students.

Panel Discussion, FSHN 199/Undergraduate Seminar in Nutrition, *2007, 2008*
- Participated in panel discussion about graduate school with freshmen human nutrition and dietetics students.